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THE EFFECT OF ADRENAL STIMULATION WITH ADRENOCORTICOTROPHIC HORMONE ON THE EXCRETION OF DEHYDROISOANDROSTERONE IN MAN[1,2,3,4,5,6]

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IN a previous paper (Landau, Knowlton, Lugibihl and Munson, 1950) we reviewed and extended the evidence relating the urinary excretion of dehydroisoandrosterone to adrenal cortical function. In the course of these studies a critical evaluation was made of the modified Pettenkofer reaction (Munson, Jones, McCall and Gallagher, 1948) employed as the means of estimating dehydroisoandrosterone and related steroids in neutral urine extracts. Since dehydroisoandrosterone and its two known hydrolytic artifacts, Δ^5 androstadienone-17 and Δ^5 3 (β) chlorandrostenone-17, possessed essentially the same chromogenic capacities and, in addition, were the only Pettenkofer chromogens which had been identified in ketonic extracts, it was felt that this procedure provided truer estimates of the

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[3] The work was aided by a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

[4] We are indebted to Dr. Edward Henderson of the Schering Corporation for dehydroisoandrosterone acetate and testosterone propionate.

[5] Chorionic gonadotrophin was generously supplied by Ayerst, McKenna, and Harrison, Ltd. (Dr. E. C. Reifenstein, Jr.).

[6] We are grateful to Dr. John Mote of Armour and Company for generous supplies of adrenocorticotrophin hormone.

dehydroisoandrosterone excreted than other methods. The chromogenicity of certain non-ketonic alcoholic steroids permitted rough approximations of this group as well.

With this procedure available, the enhanced excretion of urinary 17-ketosteroids induced by adrenal stimulation with adrenocorticotrophic hormone (Mason *et al.*, 1948; Forsham *et al.*, 1948) offered an opportunity to relate the excretion of dehydroisoandrosterone and its derivatives to different levels of activity of the normal adrenal cortex. It was hoped that by this means some clarification of the position of dehydroisoandrosterone in normal adrenal steroid metabolism might be effected. Accordingly, in these experiments we have studied the steroid excretion during periods of adrenocorticotrophic stimulation of the adrenals and compared the results with those which followed the administration of dehydroisoandrosterone acetate, testosterone propionate, and the stimulation of the testis with chorionic gonadotrophin.

METHODS

The subjects for these studies were four normal young men, university students, who lived in the metabolic ward of the hospital but pursued their usual sedentary activities, and a five year old girl with rheumatoid arthritis. The general procedure for handling such metabolic investigations and the methods for the determination of urinary nitrogen, and creatinine have been described (Kenyon, Knowlton, Sandiford, Koch and Lotwin, 1940). Urinary uric acid was determined according to Folin (1933); 17-ketosteroids were determined by a modification of the Holtorff-Koch procedure (Landau, 1949); ketonic separations, as described by Pincus and Pearlman (1941). The modified Pettenkofer reaction was performed as described by Munson, Jones, McCall, and Gallagher (1948). The materials so determined have generally been termed Pettenkofer chromogens specifying the portion of neutral urine extract assayed. The standard for both the Zimmermann and Pettenkofer reactions was dehydroisoandrosterone acetate; results are expressed as equivalents of free dehydrosioandrosterone. Eosinophil counts were performed as described by Forsham, Thorn, Prunty and Hills (1948).

All of the subjects were placed on constant adequate diets. Quantitative urine collections were made and analyzed either as twenty-four or forty-eight hour pools. Two of the men (Figs. 1 and 2) received sufficient purified adrenocorticotrophic hormone to induce characteristic metabolic responses and sharp declines in circulating eosinophil counts. The girl, E. S. (Fig. 3) received sufficient adrenocorticotrophic hormone to effect a distinct clinical improvement, early signs of Cushing's disease being noted toward the end of treatment. In a separate study the normal man, C.A. (Fig. 7) received enough dehydroisoandrosterone acetate to induce a rise in urinary 17-ketosteroids approaching the levels frequently noted in patients with adrenal cancer.

A standard six-day dosage of chorionic gonadotrophin which we had established for comparative purposes (Landau, Knowlton, Lugibihl, Brandt and Kenyon, 1950) was administered to two of the men (Figs. 4 and 5) as a means of stimulating the secretion of testicular androgen. One of them (Fig. 6) subsequently received 5 mg. per day and then 25 mg. per day of testosterone propionate, each for an eight-day period.

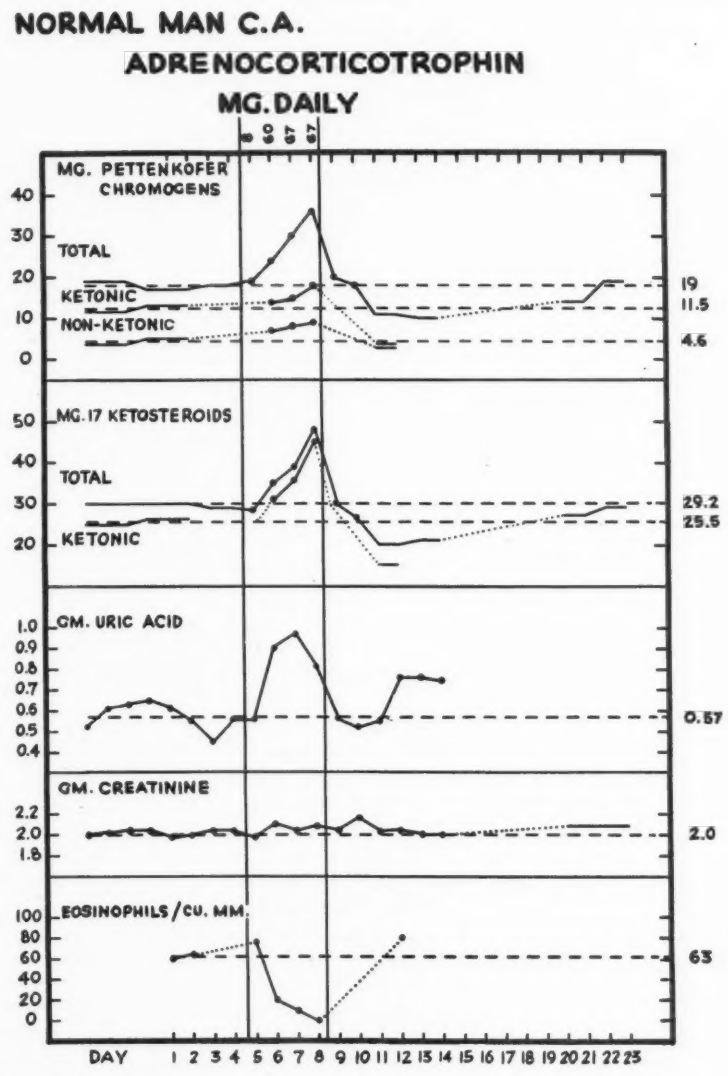

FIG. 1. The effect of adrenocorticotrophic hormone on the urinary excretion of Pettenkofer chromogens, 17-ketosteroids, uric acid and creatinine, and on the circulating eosinophils in the normal man, C.A. Broken lines represent the averages of pre-treatment control determinations. Dotted lines indicate gaps in the series of consecutive values.

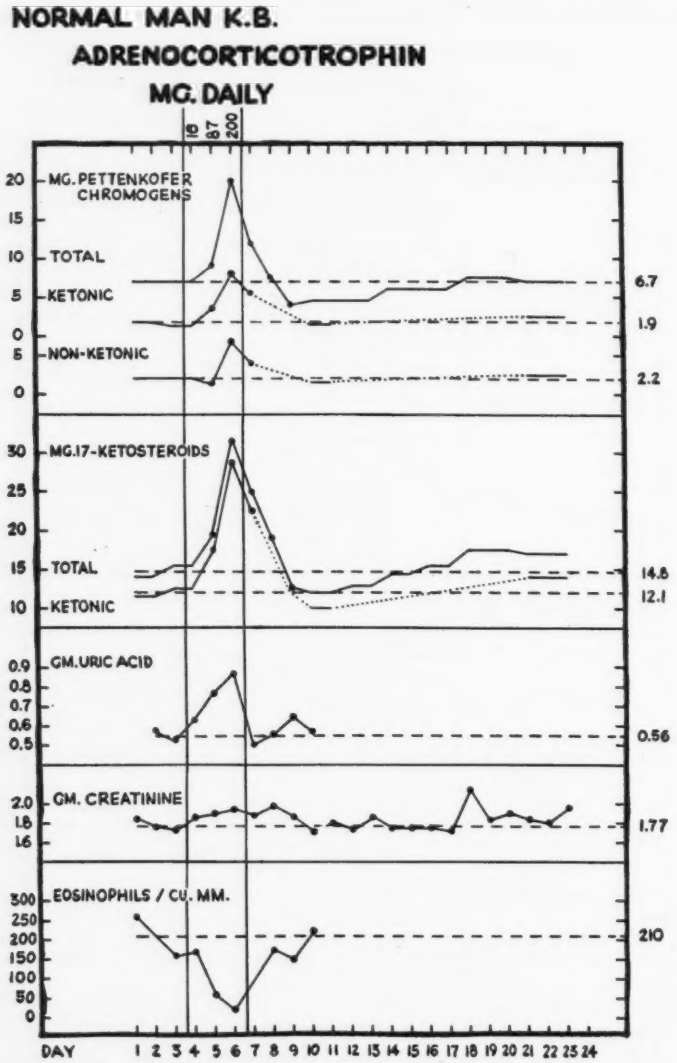

FIG. 2. The effect of adrenocorticotrophic hormone on the urinary excretion of Pettenkofer chromogens, 17-ketosteroids, uric acid and creatinine, and on the circulating eosinophils in the normal man, K.B. Broken lines represent the averages of pre-treatment control determinations. The dotted lines indicate breaks in serial determinations.

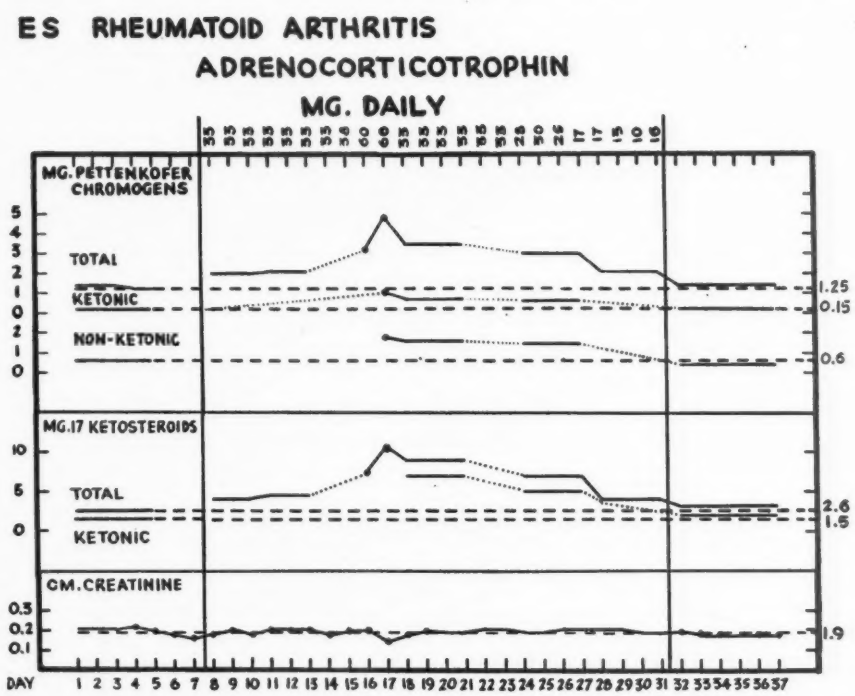

FIG. 3. The effect of adrenocorticotrophic hormone on the urinary excretion of Pettenkofer chromogens, 17-ketosteroids and creatinine in the five year old girl, E.S. The broken lines represent the averages of control values. The dotted lines indicate breaks in the series of consecutive determinations.

PROTOCOLS

C.A. was 24 years old. His basal metabolic rate was 0 (Mayo Foundation Standard). Purified adrenocorticotrophin (Armour Lot G 1012) was given for four days in the dosage noted (Fig. 1), the total administered daily being divided into equal portions and injected intramuscularly at 6 hour intervals. All quantities are based upon the assay equivalent of the Armour standard preparation LA 1 A. Later after a suitable control period dehydroisoandrosterone acetate in sesame oil was administered intramuscularly for eight days (Fig. 7). The subject felt well through both courses of treatment.

K.B. was 21 years old. His basal metabolic rate was −27 (Mayo Foundation Standard). There was no clinical evidence of hypothyroidism; the serum cholesterol was 160 mg. %. Adrenocorticotrophin was administered (Fig. 2) for three days in divided doses as in C.A. The first two days he received Armour Lot G 1012 and the last day Lot H 2403. Dosages noted are in terms of Armour standard LA 1 A. He felt well throughout.

G.M., aged 21, was without physical abnormalities; however, he was receiving occasional aid for a mild psychiatric disorder. His basal metabolic rate was −1 (Mayo Foundation Standard). He received what had been established in a study on the metabolic effects of chorionic gonadotrophin in normal young men (Landau, Knowlton,

Lugibihl, Brandt and Kenyon, 1950) as the standard course of treatment (Fig. 5). He was given chorionic gonadotrophin (A.P.L.) in two spaced injections the first day and in three divided doses the remaining five days of treatment. After the completion of this experiment 5 mg. daily of testosterone propionate was administered for eight days, followed after a three day gap by 25 mg. daily for the same period (Fig. 6).

G.J. was a 24 year old student whose basal metabolic rate was −13 (Mayo Foundation Standard). He received the standard six day dosage of chorionic gonadotrophin (A.P.L.) in the same manner as G.M. (Fig. 4).

E.S. (Fig. 3) was a five year old girl with rheumatoid arthritis whose treatment with purified adrenocorticotrophin was being studied by Dr. Albert Dorfman of the Depart-

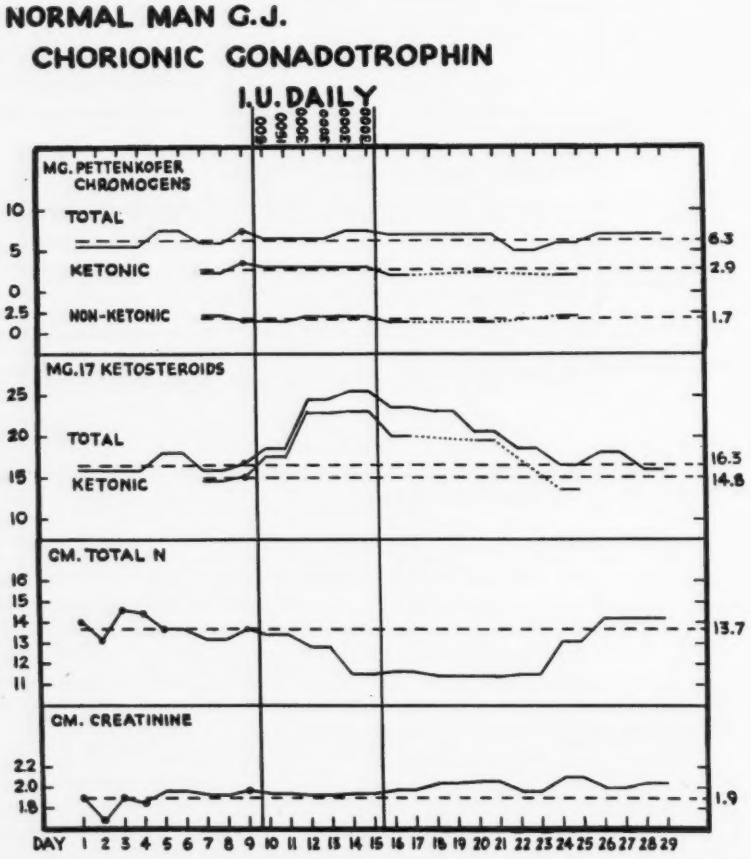

FIG. 4. The effect of chorionic gonadotrophin on the urinary excretion of Pettenkofer chromogens, 17-ketosteroids, total nitrogen and creatinine in the normal man, G.J. The broken lines represent the averages of control values. The dotted lines indicate a gap in serial determinations.

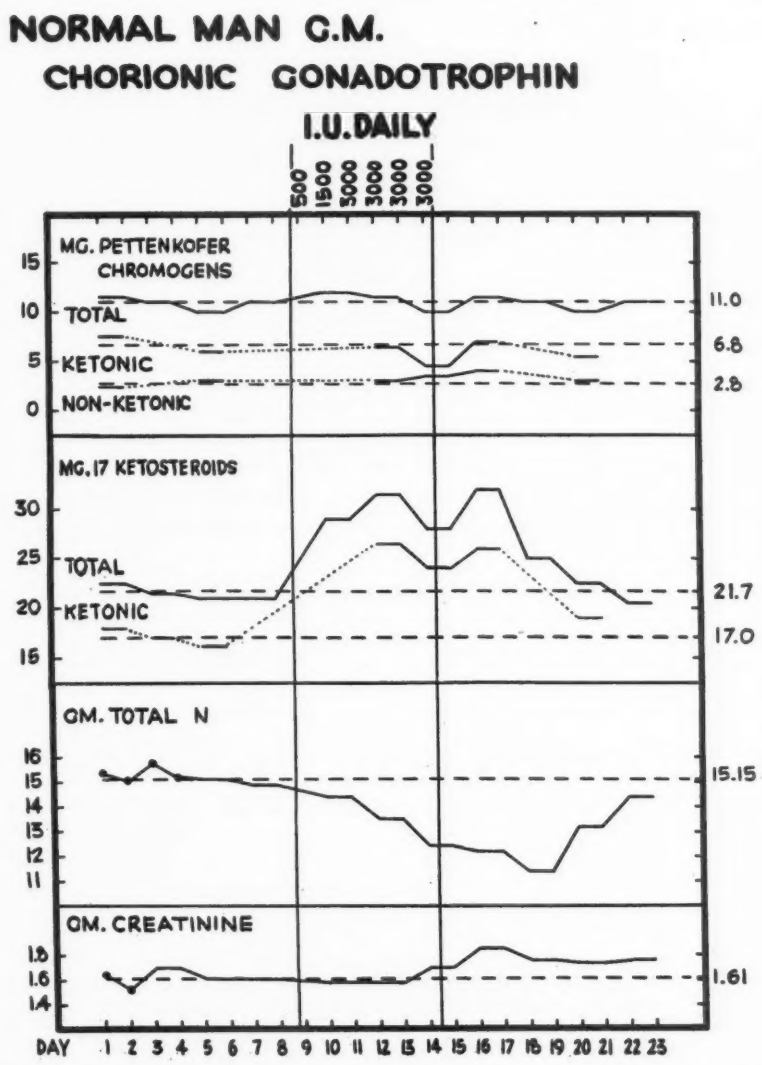

FIG. 5. The effect of chorionic gonadotrophin on the urinary excretion of Pettenkofer chromogens, 17-ketosteroids, total nitrogen and creatinine in the normal man, G.M. The broken lines represent the averages of pre-treatment controls. The dotted lines indicate gaps in serial determinations.

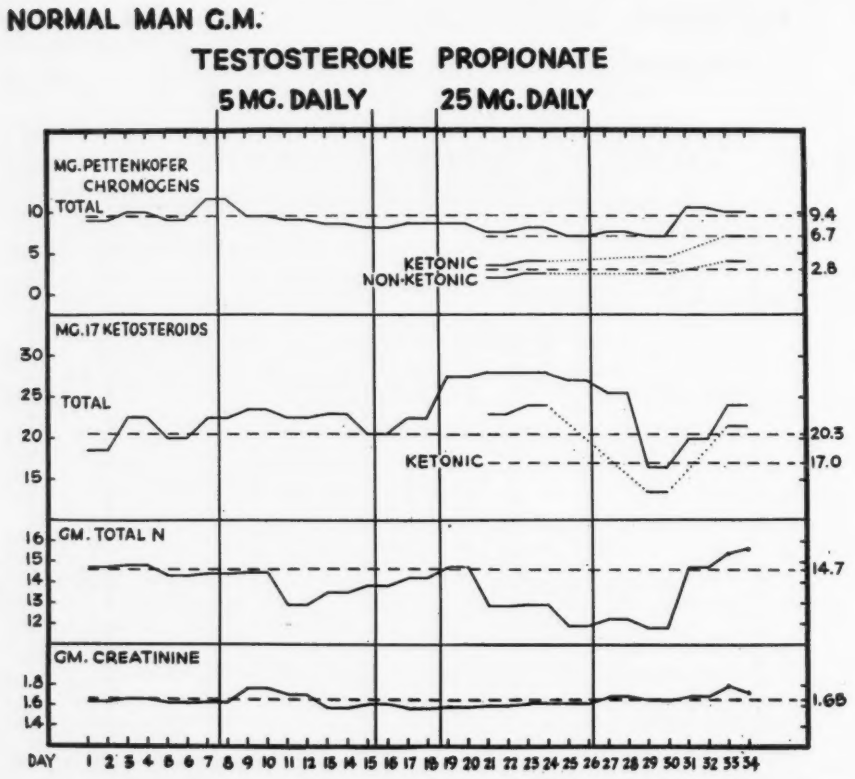

FIG. 6. The effect of testosterone propionate on the urinary excretion of Pettenkofer chromogens, 17-ketosteroids, total nitrogen and creatinine in the normal man, G.M. The broken lines represent the averages of pre-treatment control values. These baselines for the ketonic and non-ketonic fractions were obtained from the previous experiment on this subject (Fig. 5.) The dotted lines indicate gaps between determinations.

ment of Pediatrics who kindly gave his consent for the studies on ketosteroid excretion reported herein. The disease had been active for three years and was particularly severe the year prior to these studies. She was severely debilitated, bed-ridden and running an intermittent febrile course. As shown in detail in the figure the dosage of adrenocorticotrophin was increased from 33 mg. per day in four divided doses to 60 mg. daily and then rather slowly diminished before it was discontinued. Clinical improvement was distinct during the period of therapy.

RESULTS

After the administration of a test dose of 8 mg., the normal man C.A. received from 60 to 66 mg. per day of purified adrenocorticotrophin for a

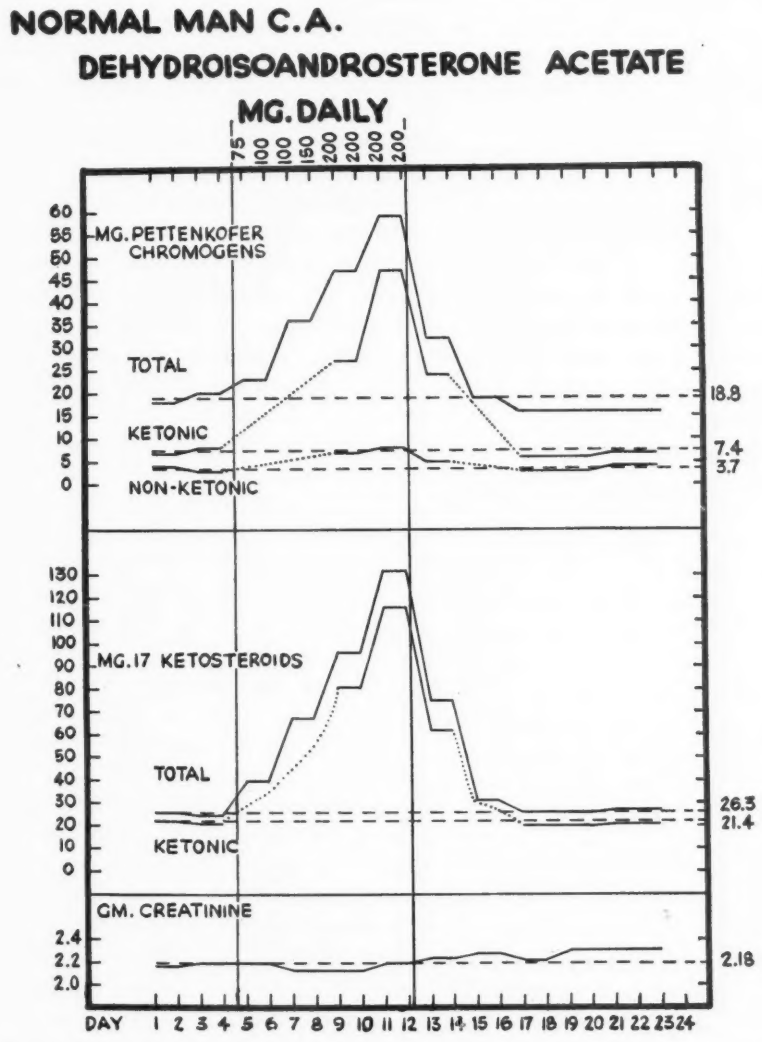

FIG. 7. The effect of dehydroisoandrosterone acetate on the urinary excretion of Pettenkofer chromogens, 17-ketosteroids and creatinine in the normal man, C.A. The broken lines represent the averages of control determinations. The dotted lines indicate gaps in serial determinations.

three-day period (Fig. 1). Ample evidence of enhanced adrenal function was noted. The eosinophil count dropped from 60/cu. mm. to none on the fourth day of treatment. On the day he received 60 mg. the urinary uric acid rose from an average excretion of 0.57 gm. per day to 0.96 gm., remaining at about that level until treatment was discontinued. The level of excretion then fell abruptly to the baseline, becoming elevated again on the 4th, 5th, and 6th post-treatment days. Total urinary nitrogen (not shown) rose slightly. No creatinuria or glycosuria was observed though two fasting blood sugars were slightly elevated. Urinary 17-ketosteroids in the crude neutral and purified ketonic extracts rose in a crescendo fashion during the last three days of adrenocorticotrophin administration, reaching a point approximately 20.3 mg. (ketonic fractions) higher than the controls, and then declined rapidly at the end of treatment reaching pre-treatment values the first day and a level 10 mg. below this baseline on the third and fourth post-treatment days. The studies were discontinued for five days, at the end of which time the 17-ketosteroid excretion had returned to the original level. The Pettenkofer chromogens followed a parallel course throughout. Their maximum elevation in the crude extract was the equivalent of 18 mg. of dehydroisoandrosterone. The ketonic chromogens rose from a pre-treatment average of 12 mg. to 18.5 mg. per day and then fell to 4 mg. the third and fourth days after treatment. The nonketonic reactors which were originally estimated at the equivalent of 4.5 mg. per day rose to 9 mg. on the last day of treatment and then declined to 3 mg.

The more intense treatment (83 and 200 mg. per day) of K.B. (Fig. 2) induced a frank diabetic state with fasting blood sugars of 156 and 154 mg. per 100 cc., the last day of treatment and subsequent day, and a glycosuria of 40 gm. the day of highest adrenocorticotrophin dosage. The excretion of urinary nitrogen was slightly elevated while, as shown on the figure, urinary uric acid rose from a baseline of 0.56 gm. per day to 0.77 and 0.87 gm. the two days of full adrenocorticotrophin administration, and immediately thereafter returned to the original level. The circulating eosinophils dropped from 163 and 169 cells per cu. mm. to 25 cells per cu. mm. at the height of treatment. Seventeen-ketosteroids in the crude neutral extracts rose from the control average of 15.0 mg. to a maximum of 31.6 mg. and had declined to 18 mg. by the second day after therapy. In the purified ketonic fractions the 17-ketosteroids rose from 12 mg. to 29.7 mg. per day, an increase of 17.7 mg. As in C.A. the Pettenkofer chromogens followed an exactly parallel course climbing from 6.7 to 19.8 mg. per day in the crude extract, from 2 to 7.9 mg. in the ketonic fraction, and from 2.2 to 6.3 mg. in the non-ketonic extract. The reflex decline in steroid excretion to levels lower than the control average was not so clearly demon-

strated in this experiment as in the first one (Fig. 1). On the fourth and fifth post-treatment days the 17-ketosteroids, total and ketonic, were from 2 to 3 mg. lower than the baseline. Although a decline of the same magnitude was indicated in the determination of the Pettenkofer chromogens in the crude extract, no very distinct parallel in the ketonic and non-ketonic fractions was evident.

In E.S. (Fig 3), the 5 year old girl with rheumatoid arthritis, the administration of adrenocorticotrophic hormone induced an initial drop in eosinophils and a satisfactory remission of the disease process. During treatment the urinary 17-ketosteroids in the crude neutral extracts rose slowly from 2.6 mg. per day to a peak of 10 mg., and declined slowly to 3.1 mg. daily as the dosage of adrenocorticotrophin was diminished. In the ketonic fractions the baseline value was 1.5 mg. per day, and the maximum value under therapy 8.4 mg. The Pettenkofer chormogens in the crude extracts closely paralleled the course of the 17-ketosteroids rising from 1.3 mg. to 4.9 mg. per day and returning to the baseline at the close of the experiment. While the actual increase in the ketonic Pettenkofer reactors was only 0.8 mg. (from 0.2 to 1.0), it is in all likelihood significant. Sufficient extract was assayed to give values within the calibration curve, and the smooth course of the decline from the maximum value lends support to the validity of the effect noted. The non-ketonic chromogens rose from 0.6 to 1.8 mg. per day.

With the exception of the data on Pettenkofer chromogens, the experimental results obtained in the studies on G.M. and G.J. (Figs. 3, 4) have been published in detail elsewhere (Landau, Knowlton, Lugibihl, Brandt and Kenyon, 1950). It was concluded that the anabolic effects induced in these and other normal men by the administration of chorionic gonadotrophin were attributable to the resultant increased secretion of testicular androgen. In order to correlate the alterations in steroid excretion with those anabolic responses, urinary nitrogen excretion values have been included in each of the figures shown here.

In G.J. (Fig. 4) the 17-ketosteroid elevation (total and ketonic fractions) accompanied the decline in urinary nitrogen, and on the third and fourth days of treatment the ketonic extracts reached a plateau which averaged 6.9 mg. higher than the controls. After eight days the 17-ketosteroid excretion declined slowly, its return to the baseline coinciding with the termination of nitrogen retention. The Pettenkofer chromogens, total ketonic and non-ketonic, were unaltered throughout.

These results were almost duplicated in the companion experiment in G.M. (Fig. 5). With the onset of nitrogen retention the 17-ketosteroids in the crude neutral extracts rose from 21.7 mg. per day to an eight-day average of 30 mg. daily, returning to the baseline just in advance of the

conclusion of the anabolic period. The ketonic fractions reached a sustained elevation of 8.5 mg. Again the excretion of Pettenkofer reacting steroids in the whole extract, as well as in both fractions, was essentially unaltered throughout.

When G.M. (Fig. 6) received 5 mg. per day of testosterone propionate, no distinct elevation in 17-ketosteroid excretion was noted. This is in accordance with expectations since the 1 to 2 mg. of 17-ketosteroids which would be derived from 5 mg. of testosterone propionate might easily be concealed in the usual day to day variations. The anabolic effect as manifested by the retention of nitrogen and other urinary constituents not charted here was transient at this dosage. The administration of 25 mg. per day of testosterone propionate resulted in a sustained increase in the excretion of 17-ketosteroids which averaged 6.4 mg. per day. After treatment the 17-ketosteroids declined sharply reaching a point 4 mg. below the control average on the third and fourth post-treatment days, and then returned to the baseline. Nitrogen retention was maintained through the 25 mg. dosage period. A slight but significant suppression of the Pettenkofer chromogens in the crude neutral extracts appears to have begun toward the end of the 5 mg. treatment period and continued until the last two days of the study. Although the level never was more than 2.5 mg. below the controls, the pattern of the decline, the absence of irregularities, and the correlation of the onset with treatment, and the return to the baseline with the termination of other testosterone effects speak for the significance of the observation. The few determinations made on ketonic and non-ketonic fractions paralleled this effect.

The administration of from 75 to 200 mg. per day of dehydroisoandrosterone acetate (total 1225 mg.) to the normal man C.A. (Fig. 7) was followed by a prompt decline of approximately 1 gm. in the excretion of urinary nitrogen (not shown on chart). However, it cannot be said that a clear-cut anabolic response was obtained since the shift in urinary nitrogen excretion was minimal and the values failed to return to the baseline at the conclusion of treatment. The 17-ketosteroids were markedly increased reaching a peak of 131 mg. per day in the crude extract. The ketonic fractions were elevated to almost the same degree. The Pettenkofer reacting steroids in the crude neutral extracts rose from 19.0 to a maximum of 58.7 mg. per day; in the ketonic fractions the parallel rise was from 7.4 to 47.0 mg. daily. By projecting the values obtained in the three treatment period extracts which were separated into ketonic and non-ketonic portions, the following calculations can be made: Approximately 480 mg. of the 1074 mg. of free dehydroisoandrosterone administered was excreted as 17-ketosteroids, and of this approximately 180 mg. or 38 per cent was accounted for by the ketonic Pettenkofer chromogens, presumably dehy-

droisoandrosterone. The non-ketonic chromogens were affected relatively slightly, increasing from the equivalent of 3.6 mg. to 7.5 mg. per day.

DISCUSSION

It is apparent that though the increased secretion of both adrenal cortical and testicular hormones is accompanied by an elevated excretion of 17-ketosteroids as measured by the Zimmermann reaction, the steroids which constitute the rise are not entirely the same in the two circumstances. In both experiments in which the testis was stimulated by chorionic gonadotrophin, the excretion of Pettenkofer reacting materials (total, ketonic, and non-ketonic) was constant through the duration of the experiment. Though dehydroisoandrosterone may be, as has been suggested by the perfusion studies of Danby (1940), a testicular intermediary in the synthesis of testosterone, no evidence of its liberation with enhanced testicular activity can be adduced from these urinary excretion studies. The rise in urinary 17-ketosteroids probably can be ascribed almost entirely to the metabolic transformation products of testicular androgen, testosterone or a near relative. Androsterone, etiocholanol-3(α)-one-17 and isoandrosterone, but not dehydroisoandrosterone, have been isolated from the urine after the administration of testosterone (Callow, 1939; Dorfman, 1941), and the absence of a rise in Pettenkofer chromogens during the administration of testosterone propionate to G.M. (Fig. 6) confirms the inability of these workers to isolate increased quantities of dehydroisoandrosterone.

From the parallel course taken by the Pettenkofer reacting steroids and 17-ketosteroids when the two normal men were treated with pituitary adrenocorticotrophin it is clear that dehydroisoandrosterone and related chromogens contribute substantially to the 17-ketosteroids of adrenal origin. On the assumption that the Pettenkofer reacting steroids in the purified ketonic fractions represent the dehydroisoandrosterone excreted, the following calculations are possible: In C.A. (Fig. 1) the aggregate three-day increase in 17-ketosteroids (ketonic fractions) was 34 mg.; at the same time the increased excretion of dehydroisoandrosterone totalled 11 mg. or 31 per cent of the 17-ketosteroids. In the study of K.B. (Fig. 2) the 17-ketosteroids during the three days of enhanced excretion totalled 30.7 mg. (ketonic fractions) in excess of the control baseline, dehydroisoandrosterone comprising 10.7 mg. or 30 per cent of the increase.

Mason and Kepler (1947) administered large quantities of dehydroisoandrosterone acetate to three patients with very low levels of 17-ketosteroid excretion (hypopituitarism and Addison's disease), and subsequently were able to isolate dehydroisoandrosterone from the urine of only one of them. In this instance the dehydroisoandrosterone equaled approxi-

mately 15 per cent of the total 17-ketosteroids. Since large amounts of androsterone and etiocholanol-3 (α)-one-17 were isolated in all three cases, it was suggested that dehydroisoandrosterone was the precursor of a large share of these steroids in normal men and women. In the similar experiment performed here on the normal man C.A. (Fig. 7) approximately 38 per cent of the increased excretion of 17-ketosteroids was calculated on the above basis to be dehydroisoandrosterone. No effort was made to identify the other derivatives, but from the studies of Mason and Kepler the presumption can be made that large amounts of androsterone and etiocholanol-3 (α)-one-17 were present.

On comparing the data obtained in the two studies on C.A. (Figs. 1 and 7) it is evident that the proportion of the increase in 17-ketosteroids determined as dehydroisoandrosterone after the administration of the acetate was very close to that accompanying adrenal stimulation. If the same rates of transformation and degradation of dehydroisoandrosterone prevailed in the two situations, almost all of the increase in 17-ketosteroids which resulted from the enhancement of adrenal function could be accounted for by the dehydroisoandrosterone liberated by the gland. Although this conclusion requires additional support, these studies strongly suggest that dehydroisoandrosterone and its metabolic and hydrolytic derivatives account for the major portion of the 17-ketosteroids of adrenal origin.

The apparent increase in non-ketonic Pettenkofer chromogens was greater in proportion to the elevation in ketonic reactors and 17-ketosteroids following adrenocorticotrophin (Figs. 1, 2) than after dehydroisoandrosterone acetate (Fig. 7). The studies of Mason and Kepler (1947) and Schneider and Mason (1948) suggest that Δ^5 androstenediol-3(β), 17(α) and androstenetriol 3(β), 16, 17 which probably contribute to the non-ketonic chromogens may be metabolic derivatives of dehydroisoandrosterone. The data presented is consistent with such a transformation of administered dehydroisoandrosterone as well as the natural product. It is possible that enhanced adrenal activity also induces an increased excretion of non-ketonic chromogenic C21 steroids such as pregnenediol-3(β), 20 (α) which has been isolated from adrenal cancer urine (Hirschman and Hirschman, 1945).

The studies in the five year old girl, E.S. (Fig. 3) demonstrated that, qualitatively speaking, the effect of adrenocorticotrophin on 17-ketosteroid excretion did not differ significantly from that noted in the men. Insufficient data is available to state that the magnitude of the enhanced 17-ketosteroid excretion in the girl was less than would be expected by a comparable dose of adrenocorticotrophin in adults, and the disproportionately low excretion of 17-ketosteroids of adrenal origin in small children (Wood and Gray, 1949), in the face of glycogenic corticoid excretion in the adult range

(Venning and Browne, 1947) remains unexplained. It must be remembered in this connection that E.S. was not a normal girl.

The studies presented here demonstrate that although the excretion of Pettenkofer chromogens may vary among individuals (Landau *et al.*, 1950), in a given subject under controlled conditions, the day to day output of these steroids is as constant as that of total 17-ketosteroids or creatinine. The maximum daily variation was no more than 15 per cent from the mean. As a consequence the enhanced excretion of Pettenkofer chromogens induced by adrenal stimulation with adrenocorticotrophin suggests that following the course of the daily excretion of these steroids may serve as a reliable means of detecting shifts in adrenal activity. This procedure may be particularly applicable for evaluating the extent of adrenal responsibility for altered 17-ketosteroid excretion in acute, complex, metabolic situations.

Thus the sustained decline in ketonic Pettenkofer chromogens from a baseline of 6.7 mg. per day to a low point of 3.3 mg. when testosterone propionate was administered to G.M. (Fig. 6) is suggestive of a suppression of adrenal cortical function. This interpretation is in agreement with the decline in the excretion of glycogenic corticoids induced by testosterone (Venning and Browne, 1947) and the reports that testosterone administration may diminish the size of the adrenal in certain laboratory animals (Parkes, 1945). The sharp post-treatment declines in the excretion of Pettenkofer chromogens and 17-ketosteroids in the two men who received adrenocorticotrophin (Figs. 1, 2) probably indicates short reflex periods of diminished adrenal activity. Such a phenomenon may be attributable to a combination of a residual depression of the secretion of adrenocorticotrophin by the subject's pituitary, and the rapid disappearance of injected material resulting in a fall in the level of circulating adrenocorticotrophin.

Though a definite relationship has been demonstrated between the secretion of adrenal cortical hormones and the liberation of dehydroisoandrosterone, its physiological and biochemical import remains obscure. The demonstration by Bloch (1945) that labeled cholesterol was converted *in vivo* to pregnandiol is an indication that progesterone, and hence perhaps other steroids are derived from cholesterol; and the disappearance of adrenal cholesterol under conditions which call forth an increased secretion of adrenal corticoids (Long, 1947) suggests that this derivation applies specifically to adrenal hormones. The fact that cholesterol and dehydroisoandrosterone possess the identical ring structures indicates that the latter may be the product of the oxidative removal of the cholesterol side chain. Thus it may be an oxidative by-product of the synthesis of C21 corticoids or an intermediary between the C21 adrenal steroids and cholesterol, the addition of the ketol side chain and oxygenation of carbon 11 taking place after removal of the aliphatic cholesterol side chain. The

inability of the isolated perfused adrenal gland to convert dehydroisoandrosterone to such hormones suggests that this second possibility does not prevail (Hechter, O. *et al.*, 1950).

Though certain 11 oxygenated urinary 17-ketosteroids are probably catabolic products of C21 adrenal steroids (Dobriner *et al.*, 1948), it is almost certain that the major portion, at least, of the dehydroisoandrosterone formed and excreted under ordinary or pathological conditions does not have such a derivation. (Huggins and Scott (1945) noted that as much as 25 mg. per day of desoxycorticosterone acetate did not influence the excretion of 17-ketosteroids in the castrated adrenalectomized man. The administration of a very large dose (200 mg. daily) of 11-dehydro-17 hydroxycorticosterone (cortisone) induced a delayed rise of only about 10 mg. per day in urinary 17-ketosteroids (Sprague *et al.*, 1950), and the inability of these workers to isolate androsterone and etiocholanol-3(α)-one-17, the principal metabolic derivatives of dehydroisoandrosterone, as components of the resulting 17-ketosteroids indicates that dehydroisoandrosterone was not derived from the injected material.

Although its liberation is clearly related to the secretion of adrenal corticoids, dehydroisoandrosterone may bear no strict biochemical relationship to the biosynthesis of these materials. It has not been isolated from adrenal extracts and no definite metabolic properties have been ascribed to it in man, but dehydroisoandrosterone may still be shown to exert as yet obscure hormonal effects.

SUMMARY

1. Using a modification of the Pettenkofer reaction as the means of determining urinary dehydroisoandrosterone and related ketonic and nonketonic chromogens, it was found that the excretion of these steroids paralleled the rise in 17-ketosteroids (Zimmermann reaction) when the normal adult adrenal cortex was stimulated with purified adrenocorticotrophic hormone. A very similar response was induced in a five year old girl with rheumatoid arthritis.

2. When an elevation in urinary 17-ketosteroids of comparable degree was induced by stimulation of the testes of normal young men with chorionic gonadotrophin, the level of excretion of dehydroisoandrosterone and other Pettenkofer reactors was unaffected.

3. A quantitative comparison of the increased excretion of ketonic Pettenkofer chromogens (dehydroisoandrosterone) and 17-ketosteroids following the administration of dehydroisoandrosterone acetate with the rise in these urinary steroids induced by adrenal stimulation in the same subject supports the suggestion that dehydroisoandrosterone and its metabolic and hydrolytic derivatives account for the major portion of the 17-ketosteroids of adrenal origin.

4. Accordingly, shifts in the daily excretion of the Pettenkofer chromogens (dehydroisoandrosterone and related compounds) in a given subject may accurately reflect alterations in adrenal activity.

5. The administration of testosterone propionate to a normal man induced a decline in the excretion of Pettenkofer reactors suggesting a slight suppression of adrenal function.

6. These results point up the desirability of ascertaining the exact significance of dehydroisoandrosterone in adrenal steroid economy.

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THE DETERMINATION OF URINARY DEHYDROISOANDROSTERONE IN NORMAL MEN AND WOMEN AND IN ADRENAL DISEASE[1,2,3,4]

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SINCE the isolation of dehydroisoandrosterone from human urine and its identification as a weakly androgenic 17-ketosteroid (Butenandt and Dannenbaum, 1934), numerous studies have suggested that it is derived directly or indirectly from the adrenal cortex. The Callows (1939) found approximately the same concentration of the steroid in the pooled urine of normal men and women and a slightly greater amount in the urine of a male castrate (1940), clearly demonstrating that its presence in urine extracts was not dependent on the testis. The fact that the administration of testosterone propionate induced a substantial increase in the excretion of androsterone and etiocholanol-3(α)-one-17 without affecting the quantity of dehydroisoandrosterone isolated (Callow, 1939), and the failure of Venning, Hoffman and Browne (1942) to isolate dehydroisoandrosterone from the urine of a man with an interstitial cell tumor of the testis and an exceptionally high excretion of 17-ketosteroids indicate that it is not derived from testicular androgen in significant quantities. There is no evidence suggesting its origin from the ovaries. On the other hand functioning tumors of the adrenal cortex are usually associated with a greatly augmented excretion of dehydroisoandrosterone (Crooke and Callow, 1939 and Engstrom, 1948). Thus although dehydroisoandrosterone has never been isolated from adrenal extracts, its urinary excretion appears to bear an intimate and exclusive relation to adrenal activity.

The present undertaking is designed to extend the description of this

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[4] We are indebted to Dr. Edward Henderson of the Schering Corporation for dehydroisoandrosterone acetate used as a standard in these studies.

relationship by employing a modification of the Pettenkofer reaction as a means of estimating the dehydroisoandrosterone excreted in the urine of normal men and women and patients with pertinent adrenal disease, as well as in normal subjects following adrenal stimulation with adrenocorticotrophin. This last part will be dealt with in the following paper (Landau, Knowlton, Lugibihl and Kenyon, 1950).

The technique used was that of Munson, Jones, McCall and Gallagher (1948). These workers, utilizing the observation of Kerr and Hoehn (1944) that dehydroisoandrosterone developed a strong blue color when treated with dilute furfural, acetic and sulfuric acids (Pettenkofer reaction), adapted a procedure for cholic acid estimation (Schmidt and Hughes, 1942) to the determination of dehydroisandrosterone. This method offers several distinct advantages. The sensitivity of the reaction (as little as 10 gamma is accurately measurable) and the ease and rapidity of performance of the procedure make it applicable to the extracts of small volumes of urine and useful for multiple serial assays.

The unique specificity of the reaction as demonstrated by Munson *et al.* (1948) is particularly fortunate. The structural requirements for a positive test appear to lie in unsaturation or potential unsaturation in ring B and in an hydroxyl group or double bond in ring A. The presence of other functional groups (ketones, alcohols) is also of importance; i.e. cholesterol (Fig. 1), which possesses the essential structure in rings A and B but has an aliphatic side chain, is not chromogenic. Of the urinary 17-ketosteroids which have been tested, only dehydroisoandrosterone, Δ^5 3(β) chlorandrostenone-17 and $\Delta^{3,5}$ androstadienone-17 give a positive reaction. Androsterone, etiocholanol-3(α)-one-17, androstanediol-3(α)-11(β)-one-17 and Δ^9 androstenol 3(α) one-17 are not chromogenic. The reactive chlorketone and dienone noted above have been identified as urinary derivatives of dehydroisoandrosterone resulting from the strong acid hydrolysis conventionally employed to split the conjugated steroids in order to facilitate their extraction by lipid solvents (Feiser and Feiser, 1949). The chromogenic capacities of dehydroisoandrosterone and Δ^5 3(β) chlorandrostenone-17 are practically the same; and when the Pettenkofer reaction is carried out at steroid concentrations in the lower portion of the standard curve, $\Delta^{3,5}$ androstadienone also coincides. Thus by means of this reaction the total of a mixture of dehydroisoandrosterone and its two known hydrolytic artifacts may be accurately measured.

Dehydroisoandrosterone has long been known to be among the most fragile of the urinary 17-ketosteroids (Gallagher, 1944). Talbot, Ryan and Wolfe (1943) noted, for example, that less than 50 per cent of sodium dehydroisoandrosterone sulfate added to urine could be recovered as dehydroisoandrosterone after boiling for ten minutes with 15 per cent

by volume of concentrated hydrochloric acid. Most of the previous estimates of urinary dehydroisoandrosterone have been dependent upon the digitonin precipitation of the 3 beta hydroxy fraction of which dehydroisoandrosterone is the principal constituent. The hydrolytic artifacts Δ^5 3(β) chlorandrostenone-17 and $\Delta^{3,5}$-androstadienone-17, pass into the alpha fraction with digitonin separations and accordingly would be missed by such a procedure. The magnitude of the conversion of dehydroisoan-

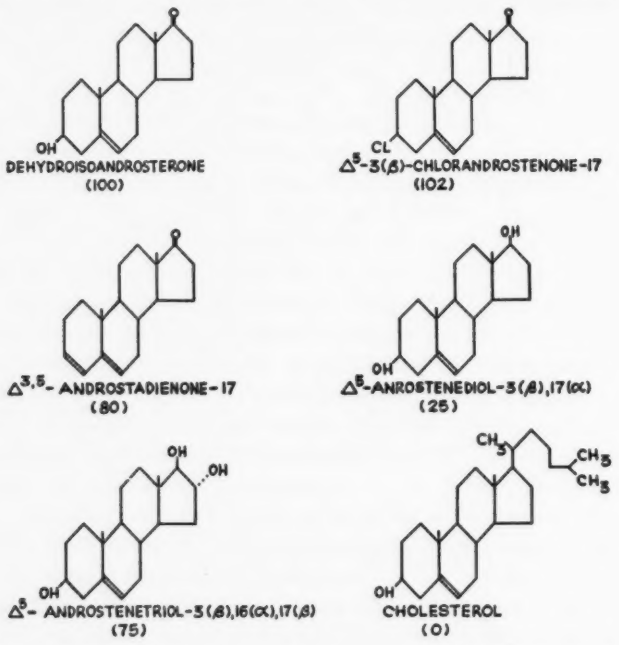

FIG. 1 Structural formulae of several pertinent steroids. Chromogenic capacities (Pettenkofer reaction) as determined by Munson *et al.* (1948) are indicated by numbers in parentheses. Dehydroisoandrosterone equals 100.

drosterone to these steroids is not precisely known but may well be substantial. Munson *et al.* (1948) found that in four of five extracts examined more than 50 per cent of the ketonic Pettenkofer reacting steroids were with the 3 alpha hydroxy fractions. Gallagher (1950) has tested all of the eluates from the chromatographic analysis of the ketonic urine extract from a normal woman, and found Pettenkofer reacting material only in those portions containing dehydroisoandrosterone and its above mentioned derivatives. Thus it is to be expected that application of the Pettenkofer reaction to ketonic extracts would give more complete and

hence higher estimates of the quantity of dehydroisoandrosterone excreted than could be expected by measurements of the beta fraction or isolation procedures with their attendant losses.

The interpretation of values obtained by the Pettenkofer analysis of non-ketonic extracts is difficult. Of the non-ketonic C19 urinary steroids Δ^5 androstenetriol-3(β), 16, 17 and Δ^5 androstenediol 3(β), 17(α) are 75 per cent and 25 per cent as chromogenic respectively as dehydroisoandrosterone (Fig. 1). The divergent reactivity of these steroids, as well as the probability that other chromogens are present in this relatively unexplored fraction, introduces confusion. The presence of these chromogens in crude neutral extracts results in falsely high estimates of their dehydroisoandrosterone content. Accordingly in order to secure a satisfactory estimate of dehydroisoandrosterone (and its two ketonic hydrolytic derivatives) the reaction must be performed on the Girard purified ketonic extract.

In an effort to avoid confusion and since it remains possible that some purified ketonic extracts as well as the non-ketonic fractions may contain unidentified chromogens, the results obtained with the modified Pettenkofer reaction will be termed Pettenkofer chromogens specifying in each case whether the crude neutral extract, ketonic or non-ketonic fraction was assayed. Although the conclusions drawn from the data which follow are based in the main upon values obtained in purified ketonic fractions, the Pettenkofer chromogens determined in crude neutral extracts may be useful as a means of indicating gross deviations and are accordingly included.

METHODS

At least two 24- hour collections of urine were made by each of the subjects whose specimens were assayed. Creatinine determinations were performed as a means of checking their completeness (Knowlton, Kenyon, Sandiford, Lotwin and Fricker, 1942). If the values were in agreement, 48- or occasionally 72-hour pools were made. Five-hundred ml. aliquots were hydrolyzed by boiling with 10 per cent by volume of concentrated hydrochloric acid. After hydrolysis the urine was cooled under running water, and the steroids were extracted by shaking with ether. The extracts were washed in the conventional manner with sodium bicarbonate and sodium hydroxide solutions to remove acidic and phenolic impurities. The ether was evaporated under reduced pressure and the residue taken up in absolute alcohol. Ketonic separations were performed as described by Pincus and Pearlman (1944); both the ketonic and non-ketonic fractions were saved. 17-ketosteroids were assayed by a modification of the Holtorff-Koch technique (Landau, 1949). The modified Pettenkofer reaction was carried out as described by Munson and his associates (1948). The standard for both procedures was dehydroisoandrosterone acetate; the results are expressed in terms of the mg. equivalent of dehydroisoandrosterone. The values are calculated on the basis of the 24-hour excretion. Those results which are starred in Tables 1 and 2 are representative of determinations performed on at least one additional pool.

THE EFFECTS OF GIRARD SEPARATION ON PETTENKOFER CHROMOGENS

Since it has been emphasized that the separation of the crude neutral extract into ketonic and non-ketonic fractions by means of the reaction with Girard's reagent T must be performed as a necessary preliminary to the estimation of the urinary dehydroisoandrosterone excreted, the limitations of the Girard treatment should be pointed out. When the extracts from normal men and women were separated into ketonic and non-ketonic fractions (Table 1), it became apparent that a portion of the Pettenkofer chromogens was lost in the process of the separation. In all cases the totals of the quantities determined in the ketonic and non-ketonic fractions failed to equal the amount

TABLE 1. PETTENKOFER CHROMOGENS AND 17-KETOSTEROIDS IN CRUDE NEUTRAL EXTRACTS, KETONIC AND NON-KETONIC FRACTIONS FROM A SERIES OF NORMAL MEN AND WOMEN

Patient	Age	Sex	Determination	Crude extract mg./24 hr.	Ketonic fraction		Non-ketonic fraction mg./24 hr.
					mg./24 hr.	% pettn. chrom.	
1	29	F	17-Ketosteroids	15.0	12.5		
			Pettn. Chrom.	5.4	2.2	17%	1.2
2	22	F	17-Ketosteroids	16.9	13.7		
			Pettn. Chrom.	3.4	1.4	10%	.8
3	23	F	17-Ketosteroids	16.6	15.7		
			Pettn. Chrom.	11.0	6.0	38%	2.0
4*	43	F	17-Ketosteroids	7.9	4.7		
			Pettn. Chrom.	7.3	3.0	63%	2.3
5	32	M	17-Ketosteroids	16.9	15.3		
			Pettn. Chrom.	6.9	2.8	18%	2.1
6*	24	M	17-Ketosteroids	16.2	14.8		
			Pettn. Chrom.	6.2	2.6	17%	1.8
7*	26	M	17-Ketosteroids	18.3	12.8		
			Pettn. Chrom.	4.6	2.1	16%	1.2
8*	21	M	17-Ketosteroids	22.7	17.9		
			Pettn. Chrom.	11.7	7.6	42%	2.6
9	24	M	17-Ketosteroids	19.5	15.6		
			Pettn. Chrom.	8.3	3.3	21%	3.9
10*	23	M	17-Ketosteroids	29.2	25.0		
			Pettn. Chrom.	19.0	12.4	49%	4.3

* Indicates values noted are representative of at least one additional analysis.

originally present in the crude extract. While a loss of about 10 per cent might be considered reasonable in view of the numerous manipulations involved, the much greater losses (28 to 37 per cent) incurred in all of the separations except the last three must be explained on another basis.

Dehydroisoandrosterone in pure solution or in the neutral extract can be quantitatively recovered in the ketonic fraction after the Girard separation; however, the other reactive steroids, ketonic and non-ketonic, have not been investigated in this manner. The fact that in Subjects 3, 5 and 6 (Table 1) this loss of Pettenkofer chromogens exceeded the difference between the 17-ketosteroids determined in the crude extracts and the amounts in the ketonic fractions suggests that in these specimens and hence probably in the others at least a portion of the materials lost was non-ketonic. For example, in Subject 3 the difference between the 17-ketosteroids in the crude extract and the ketonic fraction is 0.9 mg. Obviously no more than 0.9 mg. of ketonic material could have been lost during the separation. Since 3.0 mg. of Pettenkofer chromogens disappeared, at least a substantial portion must have been non-ketonic steroids. Until the behavior of

all the compounds concerned has been examined, however, the possibility remains that small quantities of ketonic chromogens are also lost during the Girard treatment. Thus far it does not appear that such losses seriously impair the accuracy of dehydroisoandrosterone estimates made by means of the Pettenkofer reaction.

RESULTS AND DISCUSSION

In general the Pettenkofer chromogens in crude neutral urine extracts were not closely correlated with the 17-ketosteroids. In Fig. 2 in which the Pettenkofer reacting steroids in the crude neutral urine extracts of 20

TABLE 2. PETTENKOFER CHROMOGENS AND 17-KETOSTEROIDS IN CRUDE NEUTRAL EXTRACTS, KETONIC AND NON-KETONIC FRACTIONS FROM PATIENTS WITH CUSHING'S SYNDROME, THE ADRENOGENITAL SYNDROME AND ADDISON'S DISEASE

Patient	Age	Sex	Diagnosis	17-ketosteroids mg./day		Pettenkofer chromogens mg./day		
				Crude extract	Ketonic fraction	Crude extract	Ketonic fraction	Non-ketonic fraction
L.F.*	32	F	Cushing's Syndrome (adrenal cancer)	151	146	113	78	16.8
M.G.	31	F	Cushing's Syndrome (benign adrenal tumor)	8.8		2.5		
X.P.	26	F	Cushing's Syndrome (adrenal hyperplasia)	37.9	36.5	19.6	9.8	5.2
E.P.	19	F	Cushing's Syndrome (adrenal hyperplasia)	29.2	20.8	9.1	5.5	2.7
M.M.*	15	F	Adrenogenital Syndrome (hyperplasia)	63	56.7	29.1	10.4	10.4
J.S.	7	M	Adrenogenital Syndrome (hyperplasia)	62.6	51.1	36.8	15.0	13.6
F.M.	3	F	Adrenogenital Syndrome (hyperplasia)	18.2	14.2	9.0	1.7	5.0
J.Z.	55	M	Addison's Disease	5.0	3.8	1.9	0.1	1.2
J.M.	33	F	Addison's Disease	2.9	1.9	1.1	0.1	0.6
F.M.	51	F	Addison's Disease	5.8	3.7	2.0	0.4	1.0
A.H.	46	F	Addison's Disease	2.4	1.6	0.8	0.1	0.6

* Indicates values noted are representative of at least one additional analysis.

normal men and women are plotted against the 17-ketosteroids in the same extracts, the absence of a strict relationship is graphically demonstrated. The correlation appears somewhat better in the men. Though a considerable overlap exists, some tendency for the Pettenkofer chromogens as well as the 17-ketosteroids to be higher in men is also evident. In the 9 women the excretion of Pettenkofer chromogens ranged from 2.3 to 11.0 mg. daily; the 17-ketosteroids in the same extracts, from 7.9 to 16.8 mg. per day. In the 11 men the range in the excretion of Pettenkofer reacting steroids was from 4.0 to 19.0 mg. per day while the 17-ketosteroid content of the same extracts was from 12.4 to 29.0 mg. The average excretion of Pettenkofer chromogens in the men was 8.6 mg. per day; in the women, 5.1 mg. daily.

On the assumption that the Pettenkofer chromogens determined in ketonic fractions represent the dehydroisoandrosterone excreted, 1.4 to 6.0 mg. per day of dehydroisoandrosterone was excreted by the four women and 2.1 to 12.4 mg. daily by the six men whose specimens were fractionated (Table 1). Dehydroisoandrosterone thus comprised from 10 to 63 per cent of the 17-ketosteroids (ketonic) in the women and 16 to 49 per cent in

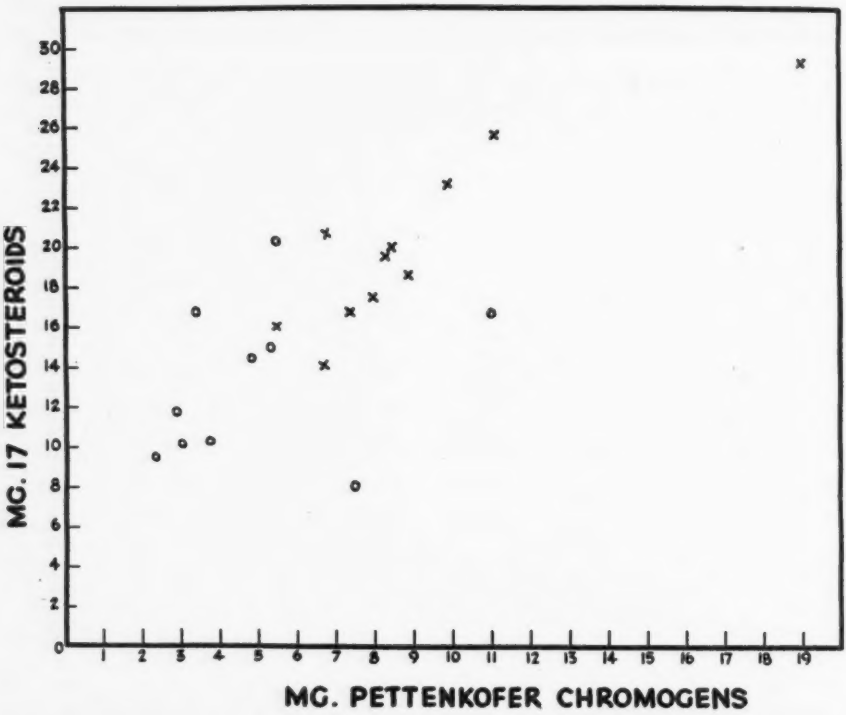

FIG. 2. Pettenkofer chromogens plotted against 17-ketosteroids in crude neutral urine extracts of normal men and women. x indicates specimens obtained from men. ○ indicates specimens obtained from women.

the men with an over-all average of 28 per cent. It should be noted that the unusually high percentages of ketonic Pettenkofer chromogens (38 to 63 per cent) were obtained in two men and two women, and that the values in the other six subjects were grouped at a level well below this (10 to 21 per cent). The highest proportion of dehydroisoandrosterone (63 per cent) was noted in a 43-year-old woman whose ketonic 17-ketosteroid excretion was only 4.7 mg. daily.

These proportions are considerably higher than the 5 to 15 per cent

frequently stated as obtaining in normal adults (Talbot, Butler, MacLachlan and Jones, 1940 and Engstrom, 1948). Most of such values, however, have been obtained by determination of the beta hydroxy fractions, actual isolation yielding even smaller quantities (Dobriner, Gordon, Rhoads, Lieberman and Fieser, 1942). Due to the lability of dehydroisoandrosterone Munson, Gallagher and Koch (1944) considered such percentages minimal at best, and the results of Talbot, Ryan and Wolfe (1943) secured by butanol extraction of the conjugates and the relatively benign barium chloride hydrolysis appear to confirm this impression. Bitman and Cohen (1949) re-examined the hydrochloric acid and barium chloride hydrolyses and found that at least 11 to 16 per cent of the urinary 17-ketosteroids of normal subjects were in the beta fraction, presumably dehydroisoandrosterone. Recently Reiss, Hemphill, Gordon and Cook (1949) reported beta fractions of from 7 to 34 per cent in a series of 12 schizophrenics. The data presented here support the concept that the quantity of dehydroisoandrosterone actually excreted by normal adults may well be substantially greater than has been indicated by most previous estimates.

In contrast to the considerable variation among individuals, the excretion of Pettenkofer reacting steroids exhibited a remarkable day-to-day constancy in a given subject. In data which will be presented in detail in the paper which follows (Landau, Knowlton, Lugibihl and Kenyon, 1950) the daily excretion of Pettenkofer chromogens was followed under controlled conditions in four normal men and found to vary no more than 15 per cent from the mean. A similar regularity has been noted in several other normal men and women and one patient with the adrenogenital syndrome.

By analyzing the urine of patients with abnormalities of adrenal cortical function, it has been possible to evaluate this procedure as a means of judging the secretory activity of the adrenals. Specimens from four patients with Cushing's syndrome, three with the adrenogenital syndrome and four Addisonians were assayed (Table 2) and the values compared with those of the normal urines (Table 1).

Of the two patients with Cushing's syndrome due to adrenal tumors (Table 2) L.F. who had a malignant neoplasm of the left adrenal, presented the usual picture. Dehydroisoandrosterone as indicated by the Pettenkofer reaction made up over half of the tremendously increased excretion of urinary 17-ketosteroids. The Pettenkofer chromogens in the ketonic fraction totaled 78 mg.; 17-ketosteroids, 146 mg. It is possible that the high levels of dehydroisoandrosterone excretion in such patients may be accounted for in part by the hydrolytic conversion to dehydroisoandrosterone of Δ^5 pregnenediol -3 (β), 17-one-20 which has been found in adrenal

cancer urine; the possibility of such a transformation has been suggested by Dorfman (1948). Δ^5 pregnenediol-3 (β), 17-one-20 has not been tested, but from its structure might be expected to develop color with the Pettenkofer reaction. Other unidentified C21 and C19 chromogenic steroids may also contribute to the enhanced excretion of ketonic Pettenkofer chromogens in such patients. On the other hand, in M.B., who had a benign tumor, the excretion of 17-ketosteroids was perfectly normal with no increase in the proportion of Pettenkofer chromogens.

The two Cushing's patients with apparent adrenal hyperplasia exhibited a slight to moderate elevation in 17-ketosteroids. E.P., who was clinically in a state of almost complete remission, excreted 20. 9 mg. per day of 17-ketosteroids (ketonic) with ketonic Pettenkofer chromogens of 5.5 mg. The latter value is just within the upper range of our normal series. X.P. whose illness was quite active, excreted 36.5 mg. of 17-ketosteroids daily and a moderately increased quantity of ketonic Pettenkofer reacting steroids (9.1 mg. daily). In both cases the proportion of Pettenkofer chromogens was no greater than was noted in the normal subjects.

The absence of any suggestion of an increased excretion of Pettenkofer chromogens and 17-ketosteroids in M.B., whose Cushing's syndrome was attributable to an adrenal tumor, stands in sharp contrast with the steroid excretion of the other patients with this syndrome and with the influence of adrenal stimulation on the ketosteroid excretion of normal men. In the paper which follows (Landau, Knowlton, Lugibihl and Kenyon, 1950) we have described the prompt rise in urinary dehydroisoandrosterone (Pettenkofer chromogens) and 17-ketosteroids induced by the administration of adrenocorticotrophic hormone. The lack of a similar elevation in the excretion of dehydroisoandrosterone and 17-ketosteroids in this patient with an obvious, prolonged hypersecretion of corticoid hormones is suggestive of a unique derangement in the processes of hormone synthesis, secretion or subsequent metabolism. This finding demonstrates that the measurement of urinary dehydroisoandrosterone (ketonic Pettenkofer chromogens) as of 17-ketosteroids cannot be taken as an index of adrenal activity under all circumstances.

In all three of the patients with the adrenogenital syndrome (adrenal hyperplasia) the excretion of 17-ketosteroids was moderately elevated. Although the proportion of Pettenkofer chromogens (ketonic) was no greater than in the normal subjects, considering their age the actual quantity of these materials excreted in all three patients was also definitely increased. The pathological adrenal androgen has never been identified in such patients, but from these data its secretion appears to be associated with the enhanced excretion of dehydroisoandrosterone. Patients in whom the virilism is attributable to an adrenal cancer generally excrete tre-

mendously increased quantities of dehydroisoandrosterone and 17-ketosteroids (Engstrom, 1949).

Urinary 17-ketosteroids in the four patients with adrenal insufficiency were low as expected, and the dehydrosioandrosterone content of the extracts was almost negligible. The quantity of ketonic Pettenkofer reacting steroids was generally so small that the color developed by the largest amount of extracts used was less than that given by the weakest standard.

The non-ketonic Pettenkofer chromogens were probably increased in all of those extracts in which the Pettenkofer reacting steroids in the crude extract and ketonic fraction were elevated. Though all of these chromogens have not been identified, Δ^5 androstenediol-3(β), 17(α) and Δ^5 androstenetriol 3(β), 16, 17 which are chromogenic have been isolated from human urine (Dorfman, 1948). Mason and Kepler (1947) secured small quantities of each from urine extracts after the administration of dehydroisoandrosterone acetate, and Miller, Dorfman and Miller (1950) confirmed the conversion to Δ^5 androstenediol-3(β). 17(α). Similarly, we observed an increase in the excretion of non-ketonic Pettenkofer chromogens after the administration of this compound to a normal man (Landau *et al.*, 1950). It thus appears that these metabolic derivatives of dehydroisoandrosterone may account for a portion of the increased excretion of non-ketonic chromogens in the above patients. The small but significant amounts of non-ketonic chromogens excreted by the Addisonians indicates that some of these steroids may not be of adrenal origin.

The quantity of dehydroisoandrosterone (ketonic Pettenkofer chromogens) as of any of the 17-ketosteroids ultimately determined in urine extracts is the resultant of a number of processes which have been discussed in some detail in connection with the decline in 17-ketosteroid excretion induced by starvation (Landau, Knowlton, Anderson, Brandt and Kenyon, 1948). The relatively wide range in the excretion of dehydroisoandrosterone in normal subjects may thus be explained by quantitative variations in any of the steps from the formation of the precursor to the renal clearance of the conjugated steroid. In spite of the variations within the normal group, the excretion of Pettenkofer chromogens in the patients with enhanced adrenal activity (Cushing's syndrome and virilism) was with a single exception consistent with the clinical state. The almost complete absence of ketonic Pettenkofer chromogens in the patients with adrenal insufficiency strongly supports the concept that all of the urinary dehydroisoandrosterone is derived from the adrenal cortex.

SUMMARY

1. A modification of the Pettenkofer reaction has been employed as a

means of estimating the content of dehydroisoandrosterone and certain related steroids in a series of normal and pathological urine extracts. The specificity of the reaction is such that the only ketonic urinary steroids which have been identified as chromogens are dehydroisoandrosterone and its hydrolytic artifacts Δ^5 3(β) chlorandrostenone-17 and $\Delta^{3,5}$ androstadienone-17.

2. On the assumption that the Pettenkofer chromogens in ketonic extracts represented the dehydroisoandrosterone excreted, it was estimated that from 1.4 to 12.4 mg. per day of dehydroisoandrosterone was excreted by a series of normal men and women. The ketonic Pettenkofer chromogens thus comprised from 10 to 63 per cent of the 17-ketosteroids in these individuals. The possibility remains that a small portion of the color developed by these purified extracts was attributable to unidentified steroids. The determination of Pettenkofer chromogens in crude neutral extracts is useful for survey purposes.

3. A patient with Cushing's syndrome due to an adrenal cancer excreted exceedingly large quantities of Pettenkofer chromogens and 17-ketosteroids. A similar patient with a benign tumor excreted normal amounts of 17-ketosteroids and Pettenkofer chromogens.

4. One of the two patients with Cushing's syndrome due to adrenal hyperplasia had a moderate elevation in 17-ketosteroids and a proportionate increase in dehydroisoandrosterone. The other patient who was in an inactive stage showed a slight increase in 17-ketosteroids with the Pettenkofer chromogens at the upper range of normal.

5. The urine of three patients with adrenal virilism (hyperplasia) contained a moderate excess of 17-ketosteroids and Pettenkofer reacting steroids.

6. Patients with Addison's disease excreted almost no dehydroisoandrosterone.

7. In general the excretion of Pettenkofer chromogens fits well with other evidences of adrenal activity. Exceptionally, however, as in the subject with a benign adrenal tumor, Pettenkofer chromogens as well as total 17-ketosteroids are less than would be expected on clinical grounds.

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EFFECT OF ADRENO-CORTICAL EXTRACTS ON BONE MARROW EOSINOPHILES OF MICE[1]

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THE regulation of the numbers of blood lymphocytes seems to be under pituitary control and is mediated by way of the adrenal cortex (Dougherty and White, 1944). In human patients with intact adrenals under administration of ACTH there is a decrease in circulating eosinophiles, a decrease in lymphocytes, and an increase in neutrophiles (Forsham, 1948). Since the decrease in eosinophiles is approximately twice as great as that of lymphocytes, the eosinophiles are a more sensitive indication of adrenal cortical activity than the lymphocytes (Forsham, 1948).

The work of Speirs and Meyer (1949) has established that stresses of various kinds will produce a marked decrease in the number of circulating eosinophiles in animals with intact adrenals and in adrenalectomized animals given adrenal cortical hormones. The response reaches a maximum at four hours after injection or stimulation, followed by a return to normal numbers of cells within eight to twelve hours.

The mechanism by which the eosinophiles are decreased is not known. That the cells are not stored temporarily in some organ of the body, such as the spleen, is indicated by the fact that splenectomized animals exhibit the response (Speirs and Meyer, 1949). The possibility remains that some organ other than the spleen may be involved. The mechanism by which the number of eosinophiles is returned to normal during recovery from the response to adrenal cortical hormones has not been investigated previously. If the eosinophiles are not stored, the return of the eosinophile count to normal would seem to be due to proliferation of new cells from the hematopoietic tissues. The logical site of such proliferation would be the bone marrow, since in the adult animal the bone marrow is the main source of eosinophilic cells in the rat and mouse.

It became of interest to the authors to find out if an increased proliferation of eosinophiles does occur in the bone marrow. The present experiment

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was designed to test the hypothesis that the return of the counts of circulating eosinophiles to normal following injection of adrenal cortical hormones in adrenalectomized mice is due to proliferation and differentiation of new cells in the bone marrow and the subsequent release of the mature eosinophiles into the blood stream.

MATERIALS AND METHODS

Male mice of the C-57 Brown strain were used. All animals were approximately three months of age and weighed from 20 to 25 grams. They were fed a diet of high protein and vitamin content. After adrenalectomy a 1% saline solution replaced their drinking water and cerelose was mixed with their food. The animals were used on an average of two weeks after adrenalectomy.

Each experimental animal received a subcutaneous injection of 0.004 cc. of an extract of the adrenal glands of hogs (Lipoadrenal Cortex, Upjohn) dissolved in Sesame oil to make a total injection volume of 0.03 cc.

At the time of injection, and when sacrificed, blood samples were taken, and absolute eosinophile counts were made by the methods of Speirs and Meyer (1949).[2]

The animals were sacrificed at 2, 4, 6, 8, 12, 24, and 36 hours following injection, and bone marrow smears were made from the femur marrow. The right leg was separated from the body at the acetabulum and the femur exposed and separated from the tibia. The epiphyses were clipped off with sharp scissors and the bone from one side of the femur chipped away with fine pointed forceps. One prong of the forceps was then slipped in between the marrow and the remaining cradle of bone and the entire marrow lifted out and placed on a clean slide. The marrow was smeared between two slides, fixed for 20 minutes in absolute methyl alcohol and stained with Wright's and Giemsa blood stains.[3]

Differential bone marrow cell counts were made on 1000 to 1200 cells under oil. Since blood cell counting is frequently subject to personal error and bias, the following precautions were taken to obtain accurate and unbiased counts: 1. When the smears were made the slides were marked with three digit numbers which were arbitrarily selected. The data for each slide were kept in a notebook and not referred to until assembled and calculated. The slides were placed in a slide box so that they were completely mixed, and at the time of counting a slide was picked at random. Thus it was impossible to know from which animal the slide had been made when the count was taken. 2. When the count was made an area was selected under low power in which the cells were evenly smeared. 3. All the cells in the field were counted, under oil immersion, following the zigzag pattern employed in counting peripheral blood smears. 4. No attempt was made to count exactly 1000 cells, so there was no necessity of watching the counter, thus decreasing the possibility of unconsciously "counting toward" any given number of cells. The counts were then calculated on the basis of 1000 cells and the per

[2] The authors would like to take this opportunity to express appreciation to Dr. Robert S. Speirs for instruction in taking blood samples and making absolute eosinophile counts and for suggestions.

[3] Flood slides with Wright's for 2 minutes. Add distilled water, mix by slight tilting of slides and allow to stand for 5 to 6 minutes. Rinse with distilled water. Flood slides with Giemsa stain, 20 drops to 10 cc., for 20 minutes. Rinse with distilled water. Dry in air by standing slides on edge on paper towel.

cent change from the control count calculated by the following formula:

$$\frac{\text{average number of cells counted per 1000}}{\text{average number of cells counted per 1000 of control}} - 1 \times 100.$$

The criteria used in identification of the stages in development of blood cells have been carried over from studies of human hematopoietic tissues to those of the rat and mouse in much of the work that has been done on these forms. There is some question concerning the advisability of this tendency, since differences are present between the cells of human and rat marrow and even slight differences between those of the rat and mouse. The differences between the developing blood cells of human and between those of the rat and mouse are most marked in the neutrophile or heterophile series. The heterophile granules of the rat and mouse are either absent, except occasionally in the promyelocyte, or do not stain well with the Wright's and Giemsa stains. In addition, the metamyelocytes form from the myelocytes most frequently by the formation of a ring-shaped nucleus rather than by indentation, although the second process also takes place (Hamre, 1947). Frequently, cells as large or larger than the promyelocytes are observed, with a ring-shaped nucleus which has a much finer chromatin pattern than the smaller metamyelocytes or than the myelocytes. The place of these cells in the developmental sequence of the heterophile has not been established with certainty. Due to these difficulties in identification, and also because the emphasis of this study was on the eosinophilic cells, all cells which appeared to be in the heterophile series were included in one count.

The criteria given in the standard texts on histology and hematology for the identification of the developmental stages of the eosinophile have been followed in this study. The eosinophilic cells of the marrow of the mouse differ very little from those of the rat.

The promyelocyte is the largest cell present in the smears, and is identified by the presence of numerous non-specific azure granules in the cytoplasm, in addition to a moderate number of orange-yellow specific staining granules. The nucleus is large, usually irregular in shape, and often with large lobes. The chromatin appears as a fine network, usually more or less obscured by the superimposed cytoplasmic granules.

The eosinophile myelocyte is slightly smaller than the promyelocyte, with a more rounded nucleus of slightly heavier chromatin network. The cytoplasm is quite basophilic, with numerous specific granules but only a few non-specific ones.

The metamyelocyte nucleus is typically ring-shaped, with the ring frequently twisted upon itself to form a figure eight. Broken-ring-shaped nuclei are occasionally found, but the band type nucleus which is often present in the heterophile metamyelocyte is encountered infrequently in the eosinophile series. The chromatin appears coarser than in the myelocyte. No non-specific granules are present, but large numbers of specific granules fill the cytoplasm. They appear less distinct in the metamyelocyte and mature eosinophile, perhaps because the basophilia of the younger stages which served to outline the granules is less pronounced in the metamyelocyte and absent in the mature cell.

The mature eosinophile as seen in the bone marrow smears is identical with that of the blood. The nucleus is ring-shaped, but with indentations present in the ring which are absent in the metamyelocyte. The chromatin appears more blocked, and darker staining than in the metamyelocyte.

The stages in the development of the erythrocyte correspond to those in the rat, and all the cells in the series were included in one count.

Megakaryocytes, plasma cells, lymphocytes, and myeloblasts were encountered

infrequently in the counting of 1000 cells. The lack of an appreciable number of lymphocytes is in disagreement with the results of Endicott and Gump (1947) who found that in myelograms of C-57 Brown mice the lymphocytes were 24.2%. The total heterophile count also differed between the two studies, being 32.4% in the results reported by Endicott and Gump and 60.0% in the control animals of the present study. Better agreement was obtained in the eosinophile counts (10.0% from Endicott's work as against 15.8%) and total erythrocyte series (30.4% from Endicott's work as against 23.8%). Discrepancies in lymphocyte and heterophile counts may have resulted from differences in methods. Endicott and Gump employed the peroxidase method in dis-

TABLE 1. MYELOGRAMS OF ADRENALECTOMIZED C-57 BROWN MICE AND ADRENALECTOMIZED MICE FOLLOWING ADMINISTRATION OF ADRENAL CORTICAL EXTRACT

Cell type	Control	2 hour		4 hour		6 hour	
	Mean S.E.	Mean S.E.	% change	Mean S.E.	% change	Mean S.E.	% change
Eosinophile Promyelocytes	13± 6.2 (6)*	9± 1.8 (6)	−31	6± 1.6 (5)	−54	9± 1.4 (6)	−31
Eosinophile Myelocytes	11± 2.8 (6)	7± 2.0 (6)	−36	11± 3.7 (5)	0	16± 1.5 (6)	+45
Eosinophile Metamyelocytes	32± 3.4 (6)	9± 3.7 (6)	−72	19± 7.4 (5)	−41	30± 3.5 (6)	− 6
Mature Eosinophiles	102±21.8 (6)	91± 6.7 (6)	−11	62±34.3 (5)	−39	64± 3.3 (6)	−37

Cell type	8 hour		12 hour		24 hour		36 hour	
	Mean S.E.	% change	Mean S.E.	% change	Mean S.E.	% change	Mean S.E.	% change
Eosinophile Promyelocytes	8± 1.3 (4)	−38	6± 3.1 (5)	−85	3± 0.7 (6)	−77	2± 0.9 (4)	−95
Eosinophile Myelocytes	18± 1.4 (4)	+64	18± 1.5 (5)	+64	8± 1.3 (6)	−27	16± 1.3 (4)	+45
Eosinophile Metamyelocytes	39± 7.4 (4)	+23	40± 6.6 (5)	+25	24± 7.8 (6)	−25	26± 0.9 (4)	−19
Mature Eosinophiles	96±10.5 (4)	− 6	89±18.4 (5)	−13	60± 7.3 (6)	−41	41±14.5 (4)	−60

* Number of animals.

$$\text{Per cent change} = \frac{\text{cells per thousand in experimental}}{\text{cells per thousand in controls}} - 1 \times 100.$$

tinguishing the cells, whereas the myelograms in this study were based entirely on nuclear and cytoplasmic differentiation, using Wright's and Giemsa stains only. In addition, the control animals of this study were adrenalectomized.

RESULTS AND DISCUSSION

The bone marrow eosinophile counts for the control adrenalectomized mice and those for the adrenalectomized mice after administration of 0.004 cc. Lipo-adrenal Cortex are given in Table 1. The column headed per cent change in the table gives the per cent change in the number of eosinophiles in the injected animals when compared with the numbers in the uninjected animals. Table 2 presents the peripheral blood counts for the injected animals, and includes the per cent change in eosinophiles between the time of injection and the time of bone marrow sampling. Table 3 summarizes the statistical analysis. Figure 1 graphically presents the per

TABLE 2. PERIPHERAL BLOOD COUNTS OF ADRENALECTOMIZED C-57 BROWN MICE FOLLOWING ADMINISTRATION OF ADRENAL CORTICAL EXTRACT

Hour		Change	Hour		Change	Hour		Change	Hour		Change	Hour		Change	Hour		Change	Hour		Change
0	2	%	0	4	%	0	6	%	0	8	%	0	12	%	0	24	%	0	36	%
105	242	+130	132	18	−86	61	7	−89	80	31	−51	54	121	+124	79	31	−61	57	110	+ 93
44	88	+100	103	13	−83	54	9	−83	128	94	−27	153	178	+ 16	54	82	+52	68	307	+351
63	42	− 33	88	17	−81	48	6	−87	92	9	−90	22	105	+377	44	63	+43	42	57	+ 36
80	12	− 85	34	3	−91	147	12	−92	56	19	−66	71	158	+122	91	77	−15	26	41	+ 58
111	37	− 67	153	35	−77	82	19	−77												
						70	14	−80												
Ave. % change		− 67			−84			−85			−58			+160			+19			+136

cent change in eosinophiles from the control values as related to the time after administration of Lipo-adrenal Cortex.

A pronounced increase in myelocytes (64%) and a less pronounced increase in metamyelocytes (23%) was observed in the bone marrow between two and eight hours after injection. Such an increase in younger stages of the bone marrow cells is commonly called a "shift to the left" and is indicative of an active production of new cells by the marrow. The greatest activity of the bone marrow took place between two and eight hours after injection when the eosinophiles of the peripheral blood were decreasing, reaching their lowest level at four hours.

The myelocytes showed the most pronounced increase, which is in keeping with the general concept that in normal bone marrow division of the myelocytes is sufficient to maintain normal numbers of granulocytes in the circulating blood (Maximow and Bloom). As is generally known, under moderate stimulation division of pre-existing myelocytes is sufficient to accomplish this, but under increased stimulation production of new myelocytes from promyelocytes occurs (Maximow and Bloom). The number of

TABLE 3. STATISTICAL ANALYSIS OF MYELOGRAMS

Tests of significance were made between counts taken at two different times after injection of adrenal cortical extract. The pairs of numbers are the hours selected for comparison.

Cell type	Comparison of counts			
	Level of significance			
	50%	30–35%	2–5%	1%
Promyelocytes	2/0; 6/0; 8/0; 12/2; 12/24; 36/24	4/0; 12/0; 24/0; 36/0	36/2; 24/2	
Myelocytes	24/0	2/0; 6/0; 36/0	8/0; 12/0	12/24; 36/24; 8/2; 8/24
Metamyelocytes	6/0; 8/0; 24/0; 36/24	4/0; 12/0; 36/0; 12/24	8/2	2/0; 12/2
Mature	2/0; 4/0; 8/0; 12/0; 8/4	6/0; 24/0; 12/24	36/0; 36/24; 8/36	

promyelocytes in this study decreased as the myelocytes increased, and they remained low throughout the experimental period. However, due to the small numbers of promyelocytes encountered it is difficult to draw conclusions concerning these cells. The total number of myelocytes produced was perhaps greater than would appear from the actual counts, since the increase in meylocytes was accompanied by an increase in meta-

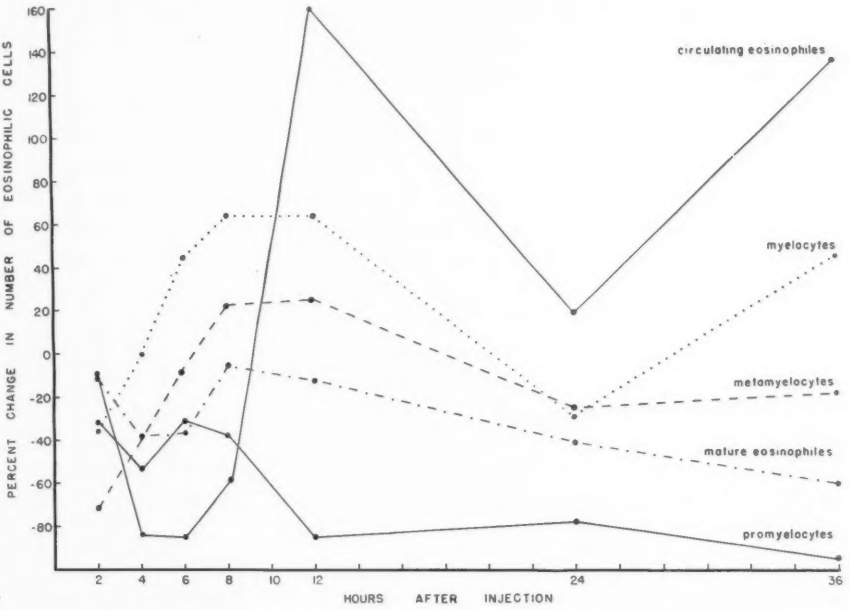

FIG. 1. Changes in bone marrow and peripheral blood eosinophiles in adrenalectomized C-57 Brown mice after administration of adrenal cortical extract.

myelocytes. It is generally agreed that metamyelocytes do not divide but come only from division of myelocytes and the subsequent differentiation of the daughter cells so produced.

There were no significant changes in the numbers of mature cells in the bone marrow during this period. Since the number of mature cells of the circulation was increasing rapidly, any mature cells produced by the bone marrow were presumably released into the circulation. The counts of mature cells showed a large standard error, which can be explained in part by the vascularity of the bone marrow, and many of the mature cells counted were cells of the circulation rather than the bone marrow.

Between 24 and 36 hours there was a significant increase in myelocytes and a significant decrease in mature cells of the bone marrow as the num-

ber of eosinophilic cells of the circulation increased. The number of metamyelocytes also increased during this period, but not over control counts, and statistical analysis proved that the increase over the counts at 24 hours was not significant. The increase in numbers of mature cells of the bone marrow during this period was the only increase that was significantly different from the numbers in the control animals. An explanation for this is not apparent from the data.

Between two and eight hours there was increased activity of the bone marrow. According to generally accepted concepts, cells which are proliferating rapidly do not undergo much cellular differentiation. Consequently, in the bone marrow during this period of rapid cell division, differentiation of cells into mature forms and the release of mature cells into the circulating blood would not occur so rapidly as in bone marrow which did not show increased activity. This could account perhaps for the decrease in numbers of mature eosinophiles of the circulation, providing the numbers present at the time of injection of adrenal cortical hormones did not remain in the circulation during the six hours following injection. It is generally accepted that the granular leucocytes as well as the lymphocytes migrate from the blood vessels into the tissues and the digestive tract (Maximow and Bloom). This may occur more rapidly under the effect of adrenal cortical hormones, or the hormone may have some effect on the eosinophiles themselves. Until these questions can be answered it is impossible to state whether the effect of adrenal cortical hormones on the eosinophiles is directly on the eosinophiles of the bone marrow or on those of the circulation, or both.

SUMMARY AND CONCLUSION

Adrenalectomized male mice of the C-57 Brown strain were injected with Lipo-adrenal Cortex and myelograms taken at 2, 4, 6, 8, 12, 24, and 36 hours after injection. There was a marked increase in younger stages of eosinophilic cells, indicating an active production of new cells by the bone marrow. The increase in activity of the bone marrow seems sufficient to explain the increase in eosinophiles in peripheral blood during recovery from the response to adrenal cortical extract.

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INHIBITION OF THYROXINE ACTION BY IODINATED PHENOXYACETIC ACIDS[1]

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INTRODUCTION

THYROXINE was first isolated by Kendall in 1915. After proof of its structure by Harington and Barger in 1927, Lyon (1927) demonstrated that upon administration of synthesized thyroxine it was possible to duplicate the action of the thyroid gland in the control of metabolic rate. The problem of thyroxine release into the blood stream has undergone an interesting evolution from 1927 to the present. Early investigators believed that the thyroglobulin or colloid was formed in the thyroid gland and entered the circulation by migrating between the follicular cells. With later developments in protein chemistry it was realized that the thyroglobulin formed in the gland must first be hydrolyzed by proteolytic enzymes, and the smaller sized molecules formed were passed into the blood stream. For some time this material was believed to be a protein, but recent work of Harington (1944), Taurog and Chaikoff (1947), Leblond and Gross (1949), Laidlaw (1949), Rall (1950) and Barker, Klitgaard and Dirks (1950) indicates that the amino acid thyroxine is found free in the circulating blood plasma. Thyroid influences on the animal body may therefore be considered to be brought about by action of thyroxine itself.

Since the isolation and synthesis of thyroxine, first interest lay in the determination of the essential constituents of the thyroxine molecule required for thyroxine-like activity (summarized by Frieden and Winzler, 1948). It was not until Fildes (1940), Woolley (1946a) and others developed the concept of specific metabolic inhibition that a second line of investigation appeared. With this theory there was presented the possibility of physiologically blocking the action of the thyroid hormone in the peripheral tissues by structurally related compounds involved in competition

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for an essential material. An elaboration of the latter was recently expressed by Harington (1948) in that it might be possible to find a compound which would act on the anterior pituitary in the same manner as thyroxine, but would lack the power of the latter to stimulate the metabolism of body tissues.

Woolley (1946b) prepared a series of ethers of N-acetyldiiodotyrosine and demonstrated that some would protect rats against a lethal dose of thyroxine as well as to interfere with the hormonal effect of thyroxine on the metamorphosis in tadpoles. No quantitative measurement of the rate of tadpole metamorphosis was reported and the degree of interference with thyroxine action could only be roughly estimated. Furthermore, it is difficult to interpret protection against lethal effects of thyroxine overdosage in terms of physiological response.

Frieden and Winzler (1949) employed a quantitative measurement of amphibian metamorphosis estimating the molar ratio of inhibitor to thyroxine required to reduce the thyroxine effect by 50 per cent. They found O-benzyl-3,5-diiodo-DL-tyrosine at a molar ratio of 37 to give 50 per cent inhibition. Three other materials tested were the benzyl, p-nitrophenylethyl, and methyl ethers of 3, 5-diiodo-4-hydroxybenzoic acid which showed inhibition at the molar ratios of 8, 75, and 150 respectively. Several of these inhibitors were active against the thyroxine-like effects of 3, 5-diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-benzoic acid but were completely inactive against 3,5-diiodo-4-(4′-hydroxyphenoxy) aniline.

Recently Maclagen, Sheahan and Wilkinson (1949) reported on the inhibitory activity of two thyroxine analogues on oxygen consumption in mice. Their results indicated that the inhibitory activity of 3,5-diiodo-4-benzyloxybenzoic acid which Frieden and Winzler observed in tadpoles was demonstrable in mice although much higher molar ratios of about 2000 were required. A second compound, 3,5-diiodoanisaldehyde dimethylacetal, had a somewhat greater antagonistic action at a molar ratio of 1100. These same investigators have made a preliminary report on more favorable results with n-alkyl-3,5-diiodo-4-hydroxybenzoates (Wilkinson, Sheahan and Maclagen, 1950).

Cortell (1949) tested seven thryoxine analogues, several containing fluorine, for anti-thyroxine activity on rats treated with thiouracil. Employing the criterion that inhibitory action would be manifested by an interference with the thyroxine-induced reduction in thyroid gland size in these animals, she found only 2′, 6′diiodothyronine to have marked effects. The other compounds, which were thyronines with varying amounts of iodine or fluorine in the 3′, 5′, 3 and 5 positions, showed small amounts of thyroxine-like activity.

Several different types of compounds were evaluated for antithyroxine

properties in three different biological testing procedures by Barker, Kiely, Dirks, Klitgaard, Wang and Wawzonek (1950). Those showing considerable amounts of activity included 3- and 4-iodophenoxyacetic acids, 2, 4-diiodophenoxyacetic acid, 3,5-diiodo-4-hydroxybenzoic acid, N-(4-hydroxy-3,5-diiodobenzoyl)-3,5-diiodotyrosine and 2-dimethylaminoethyl-dibenzofuran hydrochloride. As with mice, higher molar ratios of 4-benzyloxy-3, 5-diiodobenzoic acid were required in rats than in tadpoles.

Because of the considerable antagonistic activity found in the iodinated phenoxyacetic acids previously tested, the complete series of mono- and diiodophenoxyacetic acids was prepared for evaluation. Description of the synthesis of these compounds will be found elsewhere (Wang and Wawzonek, 1950).

EXPERIMENTAL METHODS

A Benedict multiple-chamber closed-circuit apparatus was employed to measure oxygen consumption (Benedict 1930). Since the equipment used in the investigation had four chambers, each group contained four animals. Each group of animals was allowed to equilibrate twenty to thirty minutes in the apparatus before starting the BMR. Oxygen consumption values for two consecutive thirty minute periods were obtained, and if a difference of more than 10 per cent existed a third thirty minute period was taken.

Male rats of the Sprague-Dawley albino strain, purchased directly or raised from laboratory stock, were used throughout this investigation. Thyroidectomies were performed under general ether anesthesia with at least one month allowed for recovery and full development of hypometabolism. The animals were placed on the special feeding regime described by Barker (1949) in order to have them in a post-absorptive state each day. Diet requirements were met by feeding checkers of Rockland Complete Rat Diet.

Groups of four rats were trained for several weeks and the resting metabolic rates then obtained are expressed as cubic centimeters of oxygen per hundred grams of animal weight per hour. Only animals with basal consumption of less than 75 cc. of oxygen per hundred grams per hour were used to be certain that all animals used in the experiment were totally thyroidectomized and that full hypometabolism had developed. Thyroxine[2] was then injected subcutaneously in a dose of 12μgm./kg./d. to return the metabolic rate to normal (Barker, Kiely and Lipner, 1949). The sensitivity of the thyroidectomized rat as a test animal has been discussed by Barker *et al.* (1950).

In preparing each substance for subcutaneous injection, the weighed amount was first suspended in a small volume of redistilled water. The sodium salt of the compound was then formed by the addition of 1N sodium hydroxide in small amounts to keep the final concentration below 0.02 N. If the material remained partially undissolved and mild warming did not produce a clear solution, propylene glycol was added in small quantities. Sodium chloride was used in amounts required to yield an isotonic solution.

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[2] The DL-thyroxine used in this study was generously furnished by Dr. K. W. Thompson, Organon, Inc., Orange, N. J.

GENERAL PROCEDURE

An example of results obtained is shown below:

Condition of animal	BMR
	cc. O_2/100 gm./hr.
Four untreated thyroidectomized rats	74.5
Same rats after 16 days on 12 micrograms thyroxine/kg./day	92.8
Same rats continued on thyroxine, also injected for 14 days with 3-monoiodophenoxyacetic acid (3-IPA) 2.15 mg./kg./day	82.2

Thus the average BMR of this group of thyroidectomized animals was increased by the injection of 12 μgm. of thyroxine/kg./d. from 74.5 to 92.8, a rise of 18.3 cc. of oxygen. Following the injection of the inhibitor this increase was reduced 10.6 cc. of oxygen to a BMR of 82.2. From these figures one can calculate the percentage of antagonistic activity for the 3-IPA as follows:

$$92.8-74.5=18.3$$
$$92.8-82.2=10.6$$
$$\frac{10.6}{18.3}\times 100=58.9 \text{ per cent}$$

The dose used in this study, with one exception, was equivalent to a ratio of 500 molecules of inhibitor to one of thyroxine:

$$\text{Molecular weight of 3-IPA} = 278$$
$$\text{Molecular weight of thyroxine} = 777$$
$$\frac{278}{777}=0.358$$

Therefore for 500 times 12 micrograms of thyroxine one must use 500×0.358 or 2.15 mg./kg./d.

EXPERIMENTAL RESULTS

The dose, change in oxygen uptake, and days of administration for the various iodophenoxyacetic acids will be found in Table 1. 2-Monoiodophenoxyacetic acid was found to have 21.0 per cent inhibition at 500 times, and 17.1 per cent when given at a dose of 2500 times the standard thyroxine dose. The inhibitory effect of 3-monoiodophenoxyacetic acid at 500 times isomolar was 55.5 per cent after 14 days of administration. 4-Monoiodophenoxyacetic acid was tested at 500 times isomolecular with thyroxine in two groups of animals. One group exhibited 41.5 per cent inhibition after 7 days while the other showed inhibitory activity of 57.3 per cent after the compound had been administered for 15 days.

2, 4-Diiodophenoxyacetic acid, as noted in the table, at 500 times the usual thyroxine dose showed inhibitory activity of 46.2 per cent after 14 days administration of the compound, while 2, 3-diiodophenoxyacetic acid and 6-methyl-2, 4-diiodophenoxyacetic acid were found to have no inhibitory effects.

TABLE 1. EFFECTS OF IODOPHENOXYACETIC ACIDS ON THYROXINE-INCREASED OXYGEN CONSUMPTION OF THYROIDECTOMIZED RATS

Compound			Thyroxine		BMR		Drug
Symbol	Dose*	Days	Dose	Days	Value	Change	reversal
			μg./kg./d.		cc. O_2/100 gm./hr.	cc. O_2/100 gm./hr.	%
2-IPA	0	0	0	0	75.3		
	0	0	12	13	103.9	+28.6	
	500	10	12	23	97.9	− 6.0	21.0
	2500	21	12	44	99.0	− 4.9	17.1
3-IPA	0	0	0	0	73.7		
	0	0	12	16	92.8	+19.1	
	500	14	12	30	82.2	−10.6	55.5
4-IPA	0	0	0	0	71.5		
	0	0	12	14	91.7	+20.2	
	500	7	12	21	83.3	− 8.4	41.5
	0	0	0	0	73.5		
	0	0	12	28	96.2	+22.7	
	500	15	12	43	83.2	−13.0	57.3
2,3-DIPA	0	0	0	0	75.3		
	0	0	12	13	97.9	+22.6	
	500	14	12	27	98.3	+ 0.4	− 1.8
2,4-DIPA	0	0	0	0	65.7		
	0	0	12	18	88.0	+22.3	
	500	21	12	39	77.7	−10.3	46.2
6-M-2,4-DIPA	0	0	0	0	74.6		
	0	0	12	14	94.4	+19.8	
	500	14	12	28	97.2	+ 2.8	−14.1
2,5-DIPA	0	0	0	0	71.5		
	0	0	12	14	89.1	+17.6	
	500	14	12	28	91.7	+ 2.6	−14.8
2,6-DIPA	0	0	0	0	75.3		
	0	0	12	13	97.9	+22.6	
	500	14	12	27	82.7	−15.2	67.3
4-M-2,6-DIPA	0	0	0	0	73.5		
	0	0	12	13	91.2	+17.7	
	500	14	12	27	89.9	− 1.3	7.3
3,4-DIPA	0	0	0	0	71.5		
	0	0	12	15	89.4	+17.9	
	500	14	12	29	89.5	+ 0.1	− 0.6
3,5-DIPA	0	0	0	0	74.6		
	0	0	12	18	95.5	+20.9	
	500	15	12	33	96.9	+ 1.4	− 6.7
2,4-6-TIPA†	0	0	0	0	75.3		
	0	0	12	13	96.7	+21.4	
	500	14	12	27	79.4	−17.3	80.9
5-OMe-2,4,6-TIPA	0	0	0	0	73.0		
	0	0	12	16	90.8	+17.8	
	500	14	12	30	85.1	− 5.7	32.0

* Molar ration of inhibitor: thyroxine.
† Three animals used.

No anti-thyroxine activity was found upon testing 2, 5-diiodophenoxyacetic acid at a dose of 500 times, while 2, 6-diiodophenoxyacetic acid showed marked inhibitory effect of 67.3 per cent when given at the same level for 14 days. 4-Methyl-2, 6-diiodophenoxyacetic acid was found to have only 7.3 per cent inhibition, an insignificant effect. The 3, 4- and 3, 5-diiodophenoxyacetic acids showed no inhibitory action.

The activity of 2, 4, 6-triiodophenoxyacetic acid exceeded any iodinated compound tested in this series with 80.9 per cent inhibition in 14 days at 500 times isomolecular with thyroxine, while only moderate antagonism of 32.0 per cent was found in 5-methoxy-2, 4, 6-triiodophenoxyacetic acid at the same dose. The evaluation of the former compound was calculated on the basis of only three animals since one died of infection unrelated to the experiment.

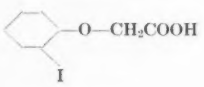
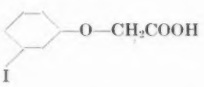
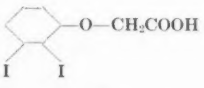
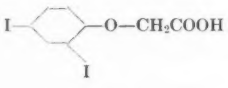
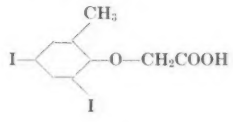
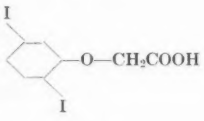
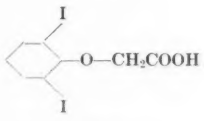
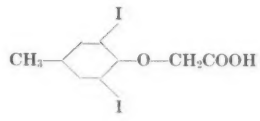
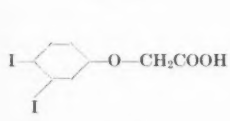
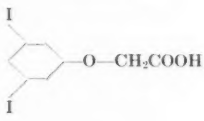
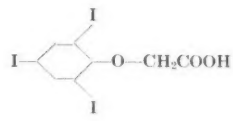
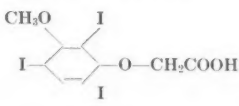
DISCUSSION

In discussing the results of this work, it has been felt best not to rely upon statistical evaluation of groups of only 4 animals each, but merely to compare results with the various iodinated phenoxyacetic acids. In addition, two groups of rats were followed for 7 and 8 weeks respectively while receiving thyroxine only to determine the fluctuations in BMR to be expected of such animals. The average variation was 16.6%. It has, therefore, seemed warranted to consider a change in metabolic rate of less than 20% to be unreliable.

Table 2 summarizes the reversals of thyroxine maintenance of BMR in thyroidectomized rats. Comparison of the structural differences and inhibitory activities of the compounds tested indicates that the 2, 4 and 6 positions are active in causing an antagonistic effect in the thyroxine-treated thyroidectomized rats. In support of this concept, 2, 4-diiodophenoxyacetic acid, 2, 6-diiodophenoxyacetic acid and 2, 4, 6-Triiodophenoxyacetic acid were found to exhibit an inhibitory effect. At 500 times the standard thyroxine dose 2, 4-diiodophenoxyacetic acid and 2, 6-diiodophenoxyacetic acid were shown to yield 46.2 and 67.3 per cent inhibition, respectively, after 14 days of administration. 2, 4, 6-triiodophenoxyacetic acid at the same dose level reversed the thyroxine response by 80.9 per cent. 4-Monoiodophenoxyacetic acid was found to have significant inhibitory activity which averaged 59.7 per cent at 500 times the thyroxine dose.

When a methyl group blocked either the 4 or 6 position of the phenyl ring of the diiodo compounds a decrease in inhibitory activity resulted. The addition of the 6-methyl group reduced the activity of 2, 4-diiodophenoxyacetic acid from 46.2 per cent to 14.1 per cent, a total decrease of 60.3 per cent. This is closely matched by the 60% drop in activity from

TABLE 2. SUMMARY OF EFFECTS OF IODINATED PHENOXYACETIC ACIDS ON REVERSAL OF THYROXINE-STIMULATED OXYGEN CONSUMPTION OF THYROIDECTOMIZED RATS

2-IPA	3-IPA	4-IPA
		
21%	56%	42%
2,3-DIPA	2,4-DIPA	6-M-2,4-DIPA
		
−2%	46%	−14%
2,5-DIPA	2,6-DIPA	4-M-2,6-DIPA
		
−15%	67%	7%
3,4-DIPA	3,5-DIPA	2,4,6-TIPA
		
−1%	−7%	81%
		5-OMe-2,4,6-TIPA
		
		32%

67.3 per cent with 2, 6-diiodophenoxyacetic acid to 7.3 per cent in 4-methyl-2, 6-diiodophenoxyacetic acid. The 2, 4, 6-triiodophenoxyacetic acid with an 80.9 per cent inhibitory effect was lowered 48.9 per cent upon the addition of a methoxy group in the 5 position. Thus, it is evident that addition of the methyl or methoxy groups resulted in a 50–70 per cent reduction of anti-thyroxine behavior in the specific compounds tested.

It is difficult to use the results with the monoiodophenoxyacetic acids

in support of the concept of 2, 4, and 6 positions being active in antagonizing thyroxine activity because of their inconsistent behavior. Although 4-monoiodophenoxyacetic acid, 2, 4-diiodophenoxyacetic acid, 2, 6-diiodophenoxyacetic acid, and 2, 4, 6-triiodophenoxyacetic acid resulted in marked depression of metabolism, 2-monoiodophenoxyacetic acid was found to have little or no effect in the thyroidectomized animals used in this experiment. This laboratory, in previous tests, found that injections of 2-monoiodophenoxyacetic acid at 109 mgm. per kilogram per day resulted in a 19 per cent anti-thyroxine response in normal rats, which effect lasted for as long as three weeks. However, this has not been confirmed by several further studies.

Diiodophenoxyacetic acids with the iodine substitutions in the 3, 4, 3, 5 and 3, 6 (2, 5) positions were shown inactive as thyroxine antagonists while the 3-monoiodophenoxyacetic acid exhibited a marked anti-thyroxine response. These results indicate some interference with the activity conferred by iodine in the 3 position when other positions in the ring are filled.

One compound tested by Cortell, the 2′, 6′-diiodothyronine, is the only one yet reported upon having halogens in any of our theoretically active positions on the outer phenyl ring. This substance was found to exhibit marked anti-thyroxine activity in thyroxinized thiouracil-treated rats at inhibitor to thyroxine ratios of about 150 to 500.

Woolley (1946b) found the p-nitrophenylethyl ether of N-acetyl-3, 5-diiodo-L-tyrosine to have marked inhibitory activity, while the p-nitrobenzyl ether of the same compound showed such an effect to a lesser degree. The N-acetyl-3, 5-diiodo-L-tyrosine itself gave no indication of having an antagonistic effect. Since the effects of these ethers might have been due to the toxicity of the nitro group, it is of interest to note that the benzyl ether, also found somewhat active by Woolley, has been further evaluated by Frieden and Winzler (1949), Maclagen *et al.* (1949) and Barker *et al.* (1950). It may be mentioned that the halogens in the 3 and 5 positions in these compounds also can be considered to be 2 and 6 with regard to the ether linkage. Thus, they too, by a stretch of nomenclature, fall into our classification. In this case, some additional characteristic must be conferred by the nature of the groups connected by the ether linkage, since 3, 5-diiodothyronine was reported by Gaddum (1930) to show a weak thyroxine-like action.

SUMMARY

A series of iodinated phenoxyacetic acids was tested for thyroxine-inhibiting action on oxygen consumption of thyroxine-treated thyroidectomized rats. Each antagonist was used at the consistent dose level of 500 times isomolar with the maintenance dose of thyroxine.

3-Monoiodophenoxyacetic acid, 4-monoiodophenoxyacetic acid, 2,4-diiodophenoxyacetic acid, 2,6-diiodophenoxyacetic acid, and 2, 4, 6-triiodophenoxyacetic acid were found to have considerable thyroxine antagonistic activity. The introduction of a methyl or methoxy group reduced the antagonistic effect of the parent compound as shown by 4-methyl-2, 6-diiodophenoxyacetic acid, 6-methyl-2, 4-diiodophenoxyacetic acid and 5-methoxy-2, 4, 6-triiodophenoxyacetic acid. 2-Monoiodophenoxy acetic acid was found to have little or no inhibitory action as was also true the following diiodophenoxyacetic acids: 2, 3, 2, 5, 3, 4 and 3, 5.

The concept is advanced that iodine in the 2, 4 and 6 positions on the phenyl ring of phenoxyacetic acid produces thyroxine-inhibiting properties. That this theory may include iodinated thyronines is supported by Cortell's finding of marked thyroxine-depressing effects of 2′, 6′-diiodothyronine.

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EFFECTS OF ADRENOCORTICOTROPIC HORMONE ON BODY GROWTH, VISCERAL PROPORTIONS, AND WHITE BLOOD CELL COUNTS OF NORMAL AND HYPOPHYSECTOMIZED MALE RATS

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THE objective of this experiment was to induce in rats as complete a growth stasis as possible by injection of adrenocorticotropic hormone, and to maintain the animals in a state of arrested growth for a relatively long period of time. The present report describes the body growth, visceral proportions, and hematology of such animals. Their skeletal morphogenesis, skeletal uptake of radio-active calcium (Ca^{45}), and chemical composition of the body will be described in subsequent papers.

In 1937 Moon demonstrated the inhibition of somatic growth in castrate rats when adrenocorticotropic pituitary extracts were administered. He also noted arrest of hair growth, and development of disproportions in visceral weight. The purification of this hormone (Li, Evans and Simpson, 1943) made possible subsequent critical studies. Marx *et al.* (1943), Evans *et al.* (1943), and Becks *et al.* (1943a and 1943b) confirmed the retardation of somatic growth, demonstrated the antagonism of this hormone to the pituitary growth hormone, and showed its capacity to retard osseous development. Li and Evans (1947) reported the inhibition of the male reproductive system in immature rats injected with ACTH. More recently the effects of administering excessive ACTH to rats have been examined by Baker and his colleagues (1948 and 1950) from the standpoint of diet, liver structure, inhibition of skin and hair, osseous development and bone marrow, and involution of the reproductive system. Another study of interest in this connection is that of Winter *et al.* (1950) in which an attempt to simulate hyperadrenocorticism was made by administration of high doses of cortisone for an extended period, the resultant growth retardation being studied both by anatomical and biochemical techniques.

There remained several questions at issue, however, which justified an additional experiment. In particular, it was sought to determine whether absolute growth stasis could be induced in otherwise normal rats, to show more clearly the consequences of ACTH-induced hypercorticism on rats

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already dwarfed through hypophysectomy, and to determine whether skeletal maturation would be altered when skeletal growth was arrested. An experiment was therefore designed with these objectives in view. In addition, studies of the terminal white blood cell and eosinophil counts, together with delineation of blood cell types presents further evidence that the normal ratio of polymorphonuclear to mononuclear leucocytes is reversed in both normal and hypophysectomized rats treated with ACTH, and also that ACTH administration is accompanied by a marked eosinopenia (Hills *et al.*, 1948, and Speirs and Meyers, 1949).

EXPERIMENTAL PROCEDURE

Male rats of the Long-Evans strain were selected when 21 days old, the day of weaning, for division into four groups: intact controls, intact rats treated with ACTH, hypophysectomized controls, and hypophysectomized ACTH-treated. The choice of this as the initial age was based on two considerations. First, for the purpose of detecting the nature of the antagonism of this hormone to growth (i.e., whether it opposes the stimulating action of growth hormone or the capacity of tissue to grow) it was desirable to use hypophysectomized rats in which some residual growth would occur following the pituitary deprival. It has been shown by Collip *et al.* (1933), Van Eck and Freud (1941), and Walker *et al.* (1950) that some growth occurs following early hypophysectomy, although it is not observed when the operation is performed subsequent to 26 days of age. Secondly, for the purpose of studies of skeletal maturation the maximum retardation practicable was sought, and it has been demonstrated that a suitable retardation occurs when hypophysectomy is performed at 21 days of age and the animals are allowed to survive until 60 days of age (Ray *et al.*, 1950, and Asling *et al.*, 1950). Hypophysectomy was therefore performed on 15 rats, and another 15 rats were retained intact.[1] Of these, eight hypophysectomized and 8 intact rats were started on the injection regimen; the remainder served as controls.

The ACTH used was prepared as described by Li *et al.* (1943). On biological assay in hypophysectomized female rats, the standard test animal, a dosage of 2 mg. per day for four days gave evidence of slight traces of interstitial cell stimulating hormone; no other anterior pituitary hormones appeared present. (Potential contamination with lactogenic hormone cannot be determined by this test.) The hormone (preparation L 2010B) was dissolved in pH 7.4 phosphate buffer, in a concentration of 2 mg ACTH per cc. The same preparation was used throughout the experiment, having been divided initially into bottles containing 3-day supplies of hormone which were refrozen to prevent deterioration and then thawed as needed. It was injected intraperitoneally on the following dosage schedule. On the 22nd day of age each animal received 1 mg. For the next 21 days the dose was 2 mg. divided and given in 2 doses, morning and evening. After this time the dosage was increased for the intact rats, which received 3 mg. per day for the next 14 days, divided into 3 daily doses spaced at 6–7 hour intervals. The hypophy-

[1] We acknowledge the kindness and skill of Miss Frances Carter in hypophysectomizing these young rats, a task made difficult not only by their small size but by their increased tendency to bleeding. Grateful acknowledgement is also made for the use of the facilities and techniques for animal care established by Dr. Herbert M. Evans in the Institute of Experimental Biology and supervised by Dr. Miriam E. Simpson.

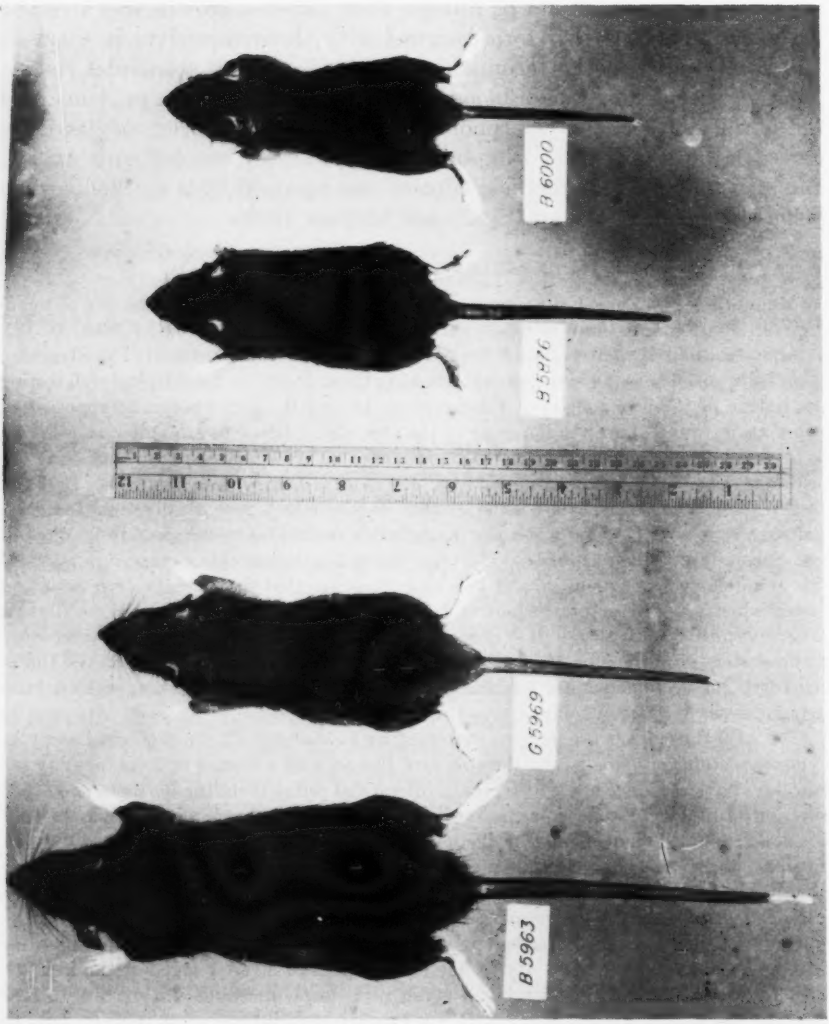

FIG. 1. Photograph at $\frac{1}{3}$ natural size showing, from left to right, a normal rat, an ACTH-injected rat, a hypophysectomized rat, and an ACTH-injected hypophysectomized rat, at 60 days of age after a 39-day injection period. Each rat was representative of its group's average length.

sectomized rats continued on the former regimen. On the 37th and 38th day, dosage was reduced for both groups to 2 mg/day for the intact rats and 1.0 mg/day for the hypophysectomized group, because weight loss appeared excessive. Finally, on the 39th day, the day of autopsy, each rat received 1 mg. of hormone in the morning. The total administered to each intact rat was 90 mg., and to hypophysectomized rats, 75 mg. The controls received no injections.

All rats were fed a moistened modified McCollum's diet (Diet I) ad lib. supplemented twice weekly with lettuce. In addition Diet XIV was always available in the cage. The animals consumed the wet diet by preference.[2] Dietary control was not exercised, since it had earlier been demonstrated that difference in food consumption could not account for the inhibiting effect of the hormone on body weight gain of intact rats (Evans *et al.*, 1943), and further that ACTH-injected hypophysectomized rats ate even more than uninjected hypophysectomized controls or hypophysectomized rats growing rapidly as a consequence of growth hormone injection (Marx *et al.*, 1943).

During the experiment all rats were weighed at a regular time of day and were observed for any signs of illness. All rats were anesthetized with sodium amytal at the mid-point of the experiment (when 41 days of age), and the day before autopsy in order to measure body length and to take roentgenographs. An hour before autopsy the animals received tracer doses of radio-active calcium. A virtually complete autopsy was performed and the remainder of the carcass retained for chemical analysis.

Blood specimens for white cell counts and differentials were taken from the tail blood of the unoperated rats on the morning prior to autopsy. Blood specimens were obtained in heparinized syringes from the inferior vena cava for counts in the operated groups. At the time of autopsy, cell counts were made with certified (USBS) pipettes and hemocytometers. Eosinophil counts were made using the phloxine diluent described by Speirs and Meyer, 1949. Eighteen squares (2 chambers) of the hemocytometer were counted. Differential counts were carried out on Wright's stained smears, 200 cells being tabulated.

RESULTS

Growth.—The most conspicuous differences which developed among the various groups were those of size, both weight and length. Figure 1 illustrates a representative rat from each group, selected on the basis of total body length at the end of the experiment. The marked stunting of the intact treated rat is apparent on comparison with the intact control. In addition, the hypophysectomized treated rat may be seen to be even more stunted than his hypophysectomized control. Figure 2 is a graph of the average body weights of rats in the four groups, and Figure 3 shows in graphic form the body lengths measured under anaesthesia at the onset, mid-point, and end of the experiment. Table 1 presents the average body weights and lengths at autopsy, and shows the gains in these measurements made during the experimental period. By reference to these graphic and

[2] Composition of Diet I: whole wheat 67.5%, casein 15%, whole milk powder 10%, iodized NaCl 0.75%, $CaCo_3$ 1.5%, hydrogenated vegetable oil 5.25% and fish oil concentrate in amount to give 1.5 chick units of vitamin D and 10.5 International Units of vitamin A per gram of diet. Composition of Diet XIV: whole wheat 68.5%, casein 5%, fish meal 10%, alfalfa leaf meal 10%, NaCl 1.5%, fish oil 5%.

tabular data it will be seen that the intact treated rats were retarded in weight gain from the beginning of the experiment. The difference between their average weight and that of the intact controls attained statistical significance on the 6th day of injections (at 27 days of age).[3] At the midpoint of the experiment (41 days of age) they corresponded in weight and

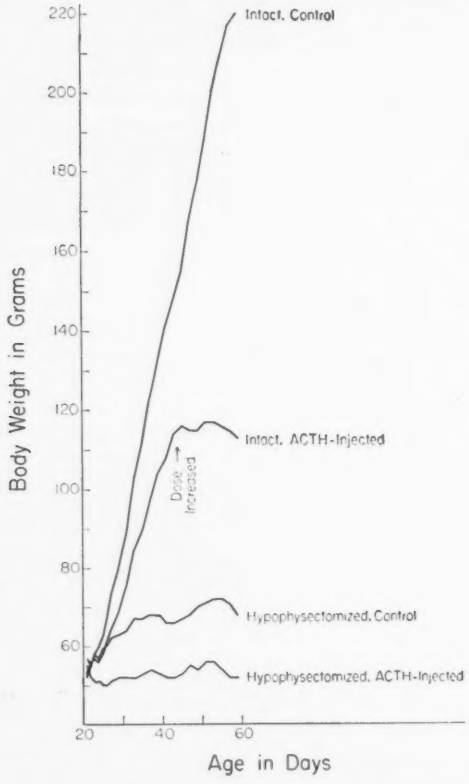

FIG. 2. Graph showing the average weights of intact and of hypophysectomized rats, uninjected and injected with adrenocorticotropic hormone from 22 to 60 days of age.

length to the dimensions which their normal controls had shown when approximately 35 days of age. During the second half of the experiment,

[3] Standard statistical methods including correction for the small numbers of animals were employed in determining standard errors. Whenever differences between values are claimed, the probability of such differences occurring by chance was less than 1% (i.e., the ratio between the difference and the standard error of that difference was greater than the t value for that number of animals in the 99% column of the t table).

when the dosage had been increased, their weight gain was negligible and their length gain only slight so that at 60 days of age they still corresponded in these respects to the normal rats at less than 38 days of age.

The hypophysectomized controls made slight (but statistically significant) weight and length gains during the first half of the experiment, and at the mid-point had body weights and lengths like those estimated for the normal controls at 26 days of age. After this time their growth was minimal, and at autopsy they had not attained the dimensions of 28-day old normal rats. The hypophysectomized treated rats, having lost weight as

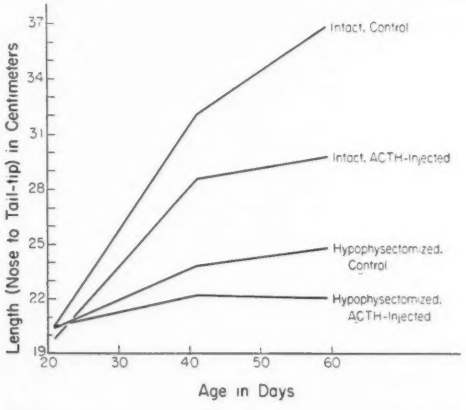

FIG. 3. Graph showing the average lengths of intact and of hypophysectomized rats, uninjected and injected with adrenocorticotropic hormone from 22 to 60 days of age. Rats were anesthetized and measured at onset, mid-point, and end of experiment.

did their controls immediately after operation, did not regain this weight[4] and at the mid-point and end of the experiment remained of the same weight as when 21 days of age. In length they attained that estimated for normal rats 23–24 days of age by the mid-point of the experiment, and then made no further gains.

Health.—The treated rats, both intact and hypophysectomized appeared less active than their controls in the latter half of the experiment. In particular, the treated hypophysectomized rats showed an asthenia. For example, it was possible to palpate small masses above the kidneys (probably their hypertrophied suprarenal glands) through the flaccid tissue of the abdominal wall.[5]

[4] The difference between the weights of hypophysectomized controls and injected hypophysectomized rats became statistically significant on the fifth day of treatment.

[5] Morbidity during the experiment was also of interest. At the mid-point of the experiment several rats in each group had upper respiratory infections (during a short

Maturation.—The pelage of the intact treated rats was only slightly retarded in development, the coarser "guard hairs" being developed as in the normal rats. The hypophysectomized treated rats retained a soft infantile pelage like their untreated controls. Observations of Moon (1937) and Baker *et al.* (1948) that ACTH retarded hair growth was confirmed by inspection of the shaved operative areas of these hypophysectomized animals. As shown in figure 4, no regeneration of hair had occurred by the time of autopsy in the treated rats.

In skeletal maturation (judged roentgenographically) the intact treated rats were neither accelerated nor retarded. The treated hypophysectomized rats were retarded to the same degree as their hypophysectomized controls

TABLE 1. AVERAGE BODY WEIGHT AND LENGTH OF 60-DAY OLD MALE RATS, INTACT AND HYPOPHYSECTOMIZED, INJECTED WITH ADRENOCORTICOTROPIC HORMONE FOR 39 DAYS

Group	No. of rats	Body weight at autopsy	Weight gain during experimental period			Body length at autopsy[1]	Length gain during experimental period		
			1st half	2nd half	Total		1st half	2nd half	Total
		gm.	gm.	gm.	gm.	cm.	cm.	cm.	cm.
Intact, control	7	220.0±5.6	87±5.0	79±4.4	167±7.9	36.9±0.6	11.5±0.4	4.8±0.2	16.3±0.5
Intact, +ACTH	5	115.0±8.6	54±5.6	7±5.6	62±9.1	29.8±0.8	8.8±0.5	1.2±0.4	10.0±0.8
Hypophysectomized, control	4	68.3±5.2	9±1.1	2±2.1	11±2.5	24.8±0.4	3.5±0.1	0.9±0.3	4.4±0.4
Hypophysectomized, +ACTH	4	51.5±2.7	−2±1.4	0±2.3	−2±3.2	22.1±0.4	1.8±0.3	−0.2±0.1[2]	1.6±0.3

[1] Body length (measured under anaesthesia) =length from nose to anus + length from anus to tail tip; see text.
[2] The value 0.2 cm. is within the error inherent in the measuring technique.

in this respect. There was some roentgenographic evidence of osteoporosis, about equally severe in the intact treated rats and the hypophysectomized controls, and rather more severe in the hypophysectomized treated rats.[6]

The testes of the majority of rats were partially descended at the onset of the experiment. In the intact rats, treated and controls, testicular descent was completed at the same rate. Regression was observed in the hypophysectomized rats after the operation, but in the treated group partial descent again occurred, with marked variation within the group. The balano-preputial cleavage was also determined, and graded on an

epidemic in the colony). After anesthesia for measurement and roentgenography this progressed to pneumonia and death in 3 of the 7 hypophysectomized controls. The treated rats made uneventful recovery. On the other hand, ACTH-injected groups, both intact and hypophysectomized, showed a definite mortality. Deaths were sudden and not preceded by any marked decline. When satisfactory autopsies could be performed, the lungs, heart, and liver appeared healthy but pale, and pleural cavities contained blood. Four rats in each injected group died. In view of the peculiar nature of these deaths, the authors do not believe it likely that their cause can be associated with the anatomical findings in the survivors.

[6] A detailed analysis of roentgenographic and histologic findings in the skeletons of these rats is in preparation.

arbitrary scale from 0 to 4+. The first evidences (1+) were seen at about 5 weeks of age in the intact controls, with complete cleavage (4+) about 10 days later. The intact treated rats followed the same course. The hypophysectomized untreated rats remained at 0 throughout the experiment. The hypophysectomized treated rats all undertook some degree of cleavage, but again marked variation was seen. A 1+ cleavage was attained by all these rats, though generally delayed until about 6 weeks

FIG. 4. Sixty-day-old rats hypophysectomized by the parapharyngeal approach. On left, a control; on right a rat after treatment for 39 days with ACTH. Note that hair has not regenerated over the shaved operative area of the treated rat, although the incision is well-healed.

of age. Some of the rats made no further progress; others attained a 3+ cleavage by about 7 weeks of age. None showed complete cleavage during the experimental period. At autopsy it was found that those which were more advanced had distinctly heavier testes.

Visceral weights and proportions.—Complete autopsies and statistical examination of organ weights, absolute and in proportion to body weight, were conducted. These data are presented in tables 2 and 3; table 2 gives mean weights and mean percentage of body weight for the major thoracic and abdominal viscera, brain and lymphatic tissues, with the standard

errors of each, and table 3 deals with the response of endocrine and reproductive organs.

The liver weight of intact treated rats, although smaller than normal, was increased in proportion to body weight. The hypophysectomized controls had livers both absolutely and proportionately smaller than normal. Hypophysectomized treated rats showed no significant differences in absolute or relative liver weights from their hypophysectomized controls.

TABLE 2. AVERAGE WEIGHTS OF VISCERA OF 60-DAY OLD MALE RATS, INTACT AND HYPOPHYSECTOMIZED, INJECTED WITH ADRENOCORTICOTROPIC HORMONE FOR 39 DAYS[1]

	Intact, control	Intact, + ACTH	Hypophysectomized, control	Hypophysectomized, + ACTH
	No. of rats			
	7	5	4	4
	gm. % BW	gm. % BW	gm. % BW	gm. % BW
Body weight	220.0 ± 5.6–	115.0 ±8.6 –	68.3 ±5.2 –	51.5 ±2.7 –
Liver	9.5 ±0.54 4.3 ±0.11	7.1 ±0.39 6.2 ±0.30	2.6 ±0.09 3.8 ±0.12	2.3 ±0.08 4.4 ±0.17
Stomach	1.15±0.04 0.52±0.01	0.66±0.05 0.58±0.03	0.44±0.02 0.64±0.03	0.42±0.04 0.82±0.03
Intestines	8.02±0.35 3.64±0.07	4.54±0.16 3.99±0.19	3.18±0.29 4.66±0.37	3.26±0.34 6.46±0.96
Heart	0.77±0.02 0.35±0.01	0.60±0.04 0.53±0.01	0.24±0.01 0.34±0.05	0 .28±0.01 0.54±0.03
Lungs	1.14±0.06 0.52±0.02	0.63±0.04 0.55±0.04	0.50±0.03 0.73±0.03	0.39±0.03 0.75±0.03
Kidneys	1.85±0.10 0.84±0.02	1.35±0.09 1.18±0.02	0.66±0.05 0.97±0.09	0.69±0.04 1.34±0.08
Brain	1.69±0.03 0.77±0.02	1.41±0.05 1.26±0.12	1.66±0.02 2.44±0.09	1.36±0.03 2.66±0.13
Spleen	1.64±0.26 0.74±0.11	0.49±0.05 0.43±0.05	0.22±0.05 0.32±0.08	0.10±0.07 0.18±0.03
	mgm. mgm. % BW	mgm. mgm. % BW	mgm. mgm. % BW	mgm. mgm. % BW
Thymus[2]	413± 38.5 186 ±13.6	59±17.9 53 ±14.4	103± 9.8 150 ±9.7	23±12.6 (3) 43 ±22.5
Cervical[2] Lymph Nodes	174± 23.2 80 ± 8.9	16± 1.2 (3) 16 ± 0.8	60± 4.8 88 ±8.8	8± 1.7 16 ± 2.7

[1] "% BW" indicates percentage of body weight; "mgm. % BW" indicates milligrams per 100 grams body weight.
[2] Numbers in parentheses indicate number of rats where entire group was not represented in average.

The stomach weight of intact treated rats was lower than normal, and remained proportionate to body weight. Stomachs of hypophysectomized controls were much smaller than normal, but were slightly increased in proportion to body weight. In hypophysectomized treated rats the stomachs were of the same weight as their hypophysectomized controls, but due to the reduced body weight they were disproportionately heavier than in any other group.

The intestines of intact treated rats weighed less than normal, remaining proportionate to body weight. Intestines of hypophysectomized controls weighed less than normal, but their proportion to body weight was not

significantly different. The hypophysectomized treated rats' intestines weighed the same as their hypophysectomized controls; although in proportion to body weight they were heavier, the small size of the groups and marked variations within groups precluded demonstration of a significant difference.

The hearts of intact treated rats weighed significantly less than normal, but were increased in proportion to body weight. The hearts of hypophysectomized controls, though absolutely smaller than normal, remained proportionate to body weight. The heart weight of treated hypophysecto-

TABLE 3. AVERAGE WEIGHTS OF ENDOCRINE AND REPRODUCTIVE ORGANS OF 60-DAY OLD MALE RATS, INTACT AND HYPOPHYSECTOMIZED, INJECTED WITH ADRENOCORTICOTROPIC HORMONE FOR 39 DAYS[1]

	Intact, control	Intact, + ACTH	Hypophysectomized, control	Hypophysectomized, + ACTH
	No. of rats			
	7	5	4	4
	mgm. mgm. % BW	mgm. mgm. % BW	mgm. mgm. % BW	mgm. mgm. % BW
Suprarenals[2]	32.0± 2.92 14.7 ± 1.02	88.0± 11.61 78.5 ±12.36	8.4± 1.29 12.3 ± 1.89	45.2± 3.32 88.2 ± 5.78
Thyroid	17.1± 2.50 7.8 ± 1.11	10.4± 0.98 9.1 ± 0.75	7.4± 0.66 10.9 ± 0.78	8.5± 2.14 16.3 ± 3.91
Pituitary[3]	7.3± 0.27 3.4 ± 0.13	4.7± 0.34 (4) 4.2 ± 0.20	— —	— —
Testis	1,320.0± 34.93 603.6 ±20.30	467.0± 79.94 399.0 ±41.60	42.0± 4.14 61.2 ± 3.83	112.0± 3.03 219.4 ±59.97
Seminal[3] Vesicle	282.3± 35.59 127.6 ±13.12	47.0± 14.9 43.3 ±15.68	6.9± 0.96 (3) 10.6 ± 1.55	14.9± 3.62 29.8 ± 8.55
Prostate[3]	304.4± 23.73 (5) 139.4 ±10.74	124.5± 25.98 (4) 109.3 ±18.29	22.4± 8.48 32.7 ±12.13	56.9± 24.05 109.4 ±44.07
Preputials[3]	127.0± 22.47 57.5 ± 8.70	103.0± 15.72 89.9 ±10.70	15.8± 2.35 23.2 ± 3.67	97.6± 16.26 (3) 185.7 ±27.16

[1] "mgm. % BW" indicates milligrams per 100 grams body weight. The standard error for all values is given.
[2] In hypophysectomized groups, one suprarenal weighed and multiplied by 2 to compensate for contralateral gland, which was removed for ascorbic acid assay.
[3] Numbers in parentheses indicate number of rats when entire group was not represented.

mized rats was the same as in their hypophysectomized controls, and was increased in proportion to body weight.

The lungs of intact treated rats were smaller than normal, remaining proportionate to body weight. The lungs of hypophysectomized controls were still smaller, but were large in proportion to body weight. Hypophysectomized treated rats' lungs were not significantly smaller than those of their hypophysectomized controls, and like them were heavy in proportion to body weight.

The weight of the kidneys of intact treated rats was smaller than normal, but large in proportion to body weight. The kidneys of hypophysectomized controls were reduced in weight, remaining proportionate to body weight. In the hypophysectomized treated group the kidney weight was the same

as in the hypophysectomized controls, and was increased in proportion to body weight.

The brain of intact treated rats was smaller than normal, and increased in proportion to body weight. Hypophysectomized controls had brains of normal weight, and were disproportionately heavy with respect to body weight. The hypophysectomized treated rats' brain weight was slightly less than the hypophysectomized controls, and showed the same disproportionate increase in percentage of body weight.

The spleen weight was absolutely and relatively smaller than normal in intact treated rats. This was also true of the hypophysectomized controls. Although the weight of spleens of hypophysectomized treated rats was still smaller, statistically significant differences from the hypophysectomized controls could not be shown either for absolute or relative weight.

The thymus of intact treated rats was reduced in weight, absolutely and relatively. It was also reduced in the hypophysectomized controls, though to a lesser degree, and not significantly in proportion to body weight. The thymus weight of hypophysectomized treated rats was markedly reduced, absolutely and relatively, from the hypophysectomized control values. The thymi in these treated animals were so small as to preclude accurate dissection and cleaning for weighing; in one rat no tissue could be found which could be identified as thymus with certainty.

The cervical lymph nodes followed the same pattern as the thymus. Here again, and in both groups of treated rats, it was difficult to identify and dissect the atrophic lymphatic tissue.

The suprarenal glands of intact treated rats were markedly heavier, absolutely and relatively, than normal. In hypophysectomized controls these glands were very small, but this reduction was in proportion to body weight. Injection of adrenocorticotropic hormone in hypophysectomized animals brought the suprarenals to greater than normal weight, and caused an even more marked hypertrophy in proportion to body weight than in the intact injected animals.

The only difference in thyroid glands' weights which developed statistically significant levels was the lighter than normal weight in hypophysectomized controls; even here the reduction remained proportionate to body weight.

The pituitary glands, although reduced in absolute weight in the intact treated rats, were large in proportion to body weight.[7]

The testes were markedly depressed in weight in intact treated rats, both absolutely and relatively. (The table gives the weight of one testis.)

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[7] Pituitary glands were weighed after fixation. Data were obtained through courtesy of Dr. A. A. Koneff, who is making cytologic studies on this tissue.

The atrophic testes of the hypophysectomized controls were also light in proportion to body weight. In hypophysectomized treated rats the results were equivocal; the average testicular weight and the ratio of testis weight to body weight, although obviously higher than in the hypophysectomized controls, did not show statistically significant differences. Great individual variation was found in this treated group, the testis weights of two of the animals (156 and 170 mg.) being distinctly heavier than those usually found in hypophysectomized animals, although even then only one-eighth the weight of the normal controls' testes.[8] The other two animals

TABLE 4. WHITE BLOOD CELL COUNTS OF 60-DAY OLD MALE RATS, INTACT AND HYPOPHYSECTOMIZED, TREATED WITH ADRENOCORTICOTROPIC HORMONE FOR 39 DAYS

Group	Number of animals	WBC thousands/cu. mm.	% Polymorphonuclear leucocytes	% Mononuclear leucocytes	Eosinophilic† leucocytes number/cu. mm.
Intact, untreated	7	14.1* (8.15–18.8)	14.6 (10–30)	85.4 (70–90.5)	152 (88–240)
Intact+ACTH	4	7.67 (7.2–8.3)	89.5 (83.5–93)	10.5 (7–16.5)	3 (0–11)
Hypophysectomized	4	3.61 (1.5–9.2)	13‡ (5–26)	87‡ (74–95)	38 (22–77)
Hypophysectomized +ACTH	4	5.07 (2.6–7.1)	79 (69–90.5)	21 (9.5–22)	0 (0)

* Average values, with ranges in parentheses.
† Direct counting method.
‡ 3 rats.

had values within the usual range (45 and 76 mg.) for hypophysectomized rats.

The seminal vesicles and prostate glands followed exactly the same pattern as the testes.

The preputial glands of intact treated rats did not differ significantly from normal control weights. In hypophysectomized controls these glands were reduced in weight, absolutely and relatively. In the hypophysectomized treated rats they were increased in weight, both absolutely and relatively.

White blood cell counts.—Table 4 presents the pertinent data on the changes in total white blood cell count, direct eosinophil counts, and on the differential white cell count, cells being classified as either mononuclear (lymphocytes and monocytes) or as polymorphonuclear leucocytes. It

[8] In view of the known presence of traces of interstitial cell stimulating hormone in the preparation used, this contaminant, rather than an assumed evocation of suprarenal androgen secretion, must be held accountable for such responses.

will be noted that after chronic administration of ACTH, the respective ratios of mononuclear/polymorphonuclear cells are reversed in the treated groups, and that this reversal is accompanied by a marked eosinopenia. Confirmation is also offered for the finding of Speirs and Meyer '49 that the eosinophil leucocyte count in hypophysectomized animals is lower than in intact controls.

SUMMARY

Male rats of the Long-Evans strain, both intact and hypophysectomized, were injected for 39 days (from the 22nd to the 60th day of age) with adrenocorticotropic hormone. Growth was retarded in the intact rats by a dosage of 2.0 mg. daily, and brought to a stop by 3.0 mg. daily. The hypophysectomized rats were even more stunted than their controls with a dosage of 2.0 mg. daily. Some visceral disproportions to body weight developed. In the intact treated rats the liver, heart, kidneys, brain, pituitary and suprarenals were disproportionately heavy, and the spleen, thymus, cervical lymph nodes, testes, seminal vesicles, and prostate were disproportionately light. In the hypophysectomized treated rats the stomach, intestines, heart, lungs, kidneys, brain, and suprarenals were disproportionately heavy, and the spleen, thymus, and cervical lymph nodes disproportionately light. (The reproductive system in the hypophysectomized treated rats was disproportionately light as in the hypophysectomized controls.) Regeneration of hair in shaved areas did not occur in the hypophysectomized treated rats. As judged by roentgenograms, skeletal maturation was neither accelerated nor retarded by the treatment. Confirmation is presented for the lymphocytopenic and eosinopenic action of ACTH as seen in chronic administration of ACTH.

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THE EFFECT OF SEX HORMONES ON HEPATIC AND RENAL LESIONS INDUCED IN RATS BY A CHOLINE-DEFICIENT DIET[1,2]

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INTRODUCTION

WHILE producing cirrhosis in rats by high fat, low choline diets, the authors observed that the livers of female rats showed less severe lesions than those of males. Similarly, the kidneys of young females appeared to withstand the effects of low choline diets better than the kidneys of young males. These findings are in agreement with those first reported by Griffith (1940).

In an effort to explore the factors involved in these sex related differences, injections of estrogens and androgens were given to groups of both males and females on the experimental diet. The results indicate that the renal and hepatic lesions which develop after feeding rats a high fat, low choline diet may be altered by hormonal injections. The present findings, plus those reported in a preliminary note (1947) complement the work of Gyorgy, Rose, and Shipley (1947, 1949). They found that estrogens had a lipotropic effect when administered to intact and castrated female rats over a 21 day period. The present findings are also consonant with the report of Ahumada and co-workers (1945) that on a high fat diet, female rats lived almost twice as long as male rats before dying of fatty livers. The interesting finding of the present experiments is that although estrogens appear to have an ameliorating effect on the production of dietary cirrhosis, androgens have an aggravating effect.

One hundred and forty-five Harvard Colony rats were used.[3] Forty-eight were 8 month old males, weighing from 222 gm. to 334 gm.; 97 were females, weighing between 64 gm. and 130 gm. Both the males and the females were divided into 6 evenly matched groups.

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[1] This work was supported by grants from the American Cancer Society and from the Donner Foundation.

[2] This is reprint 733 of the Cancer Commission of Harvard University.

[3] These rats were obtained originally from Sprague-Dawley and have been inbred periodically in Hisaw's Laboratory by brother and sister matings during the last 15 years.

Group & sex		Number of animals	Diet	Hormone
F	♀	18	high fat, low choline	none
FE	♀	25	high fat, low choline	estradiol dipropionate
FA	♀	24	high fat, low choline	testosterone propionate
C	♀	10	control stock*	none
CE	♀	10	control stock	estradiol dipropionate
CA	♀	10	control stock	testosterone propionate
F	♂	10	high fat, low choline	none
FE	♂	10	high fat, low choline	estradiol dipropionate
FA	♂	10	high fat, low choline	testosterone propionate
C	♂	6	control stock	none
CE	♂	6	control stock	estradiol dipropionate
CA	♂	6	control stock	testosterone propionate

* The control stock diet was Eschelman's Red Rose Dog and Puppy Food.

The low choline diet (subsequently called diet F) was modified after Gyorgy (1939). Each 100 gm. of the diet contained:

casein*	10 gm.
hydrogenated cotton seed oil*	20 gm.
glucose*	63 gm.
salt mixture (Webster, 1942)	5 gm.
cystine	1 gm.
cholesterol	1 gm.
fortified corn oil*	0.14 gm.
thiamin	200γ
riboflavin	400γ
pyridoxine hydrochloride	200γ
niacin	2500γ
calcium pantothenate	1500γ

* The casein was Labco brand, alcohol extracted "vitamin-free." The hydrogenated cotton seed oil was "Crisco," and the glucose was "Cerelose." Each 100 cc. of the corn oil contained 1.33 gm. haliver oil, 0.66 gm. viosterol, and 1.0 gm. a-tocopherol.

The animals were fed the diet from the beginning of the experiment until they died or were sacrificed. For the females, the feeding period extended from 7 to 234 days; for the males, from 101 to 143 days. The food consumption of all groups of rats on the special diet was measured and the average daily intake calculated.

Since many young female rats died soon after the institution of this diet, 10 mg. choline chloride was injected into each of the remainder on the 7th day as an emergency measure. Thereafter, choline chloride was added to the drinking water or to the diet in amounts calculated to give each female 1 mg. choline chloride per day when the average daily consumption of water and food was 12.5 cc. and 10 gm. respectively. After the third month, this supplement was reduced to 0.5 mg. a day per rat, incorporated in the diet. The choline was dissolved in water, rapidly emulsified with the melted fat, and added to the dry ingredients. Such a supplement was not necessary for the adult male rats.

The hormones were administered subcutaneously. The dosage was the same for both the males and the females. All animals receiving estrogen were given 0.20 mg. estradiol dipropionate in sesame oil twice weekly; those given androgen were injected twice weekly with 2.5 mg. testosterone propionate, in sesame oil.[4] For convenience in measurement, both hormones in sesame oil were diluted with corn oil.[5] Thus, the animals given hor-

[4] The hormones used in this experiment were generously supplied by Ciba Pharmaceutical Products, Inc.

[5] Sesame oil was not available at the time when this experiment was started.

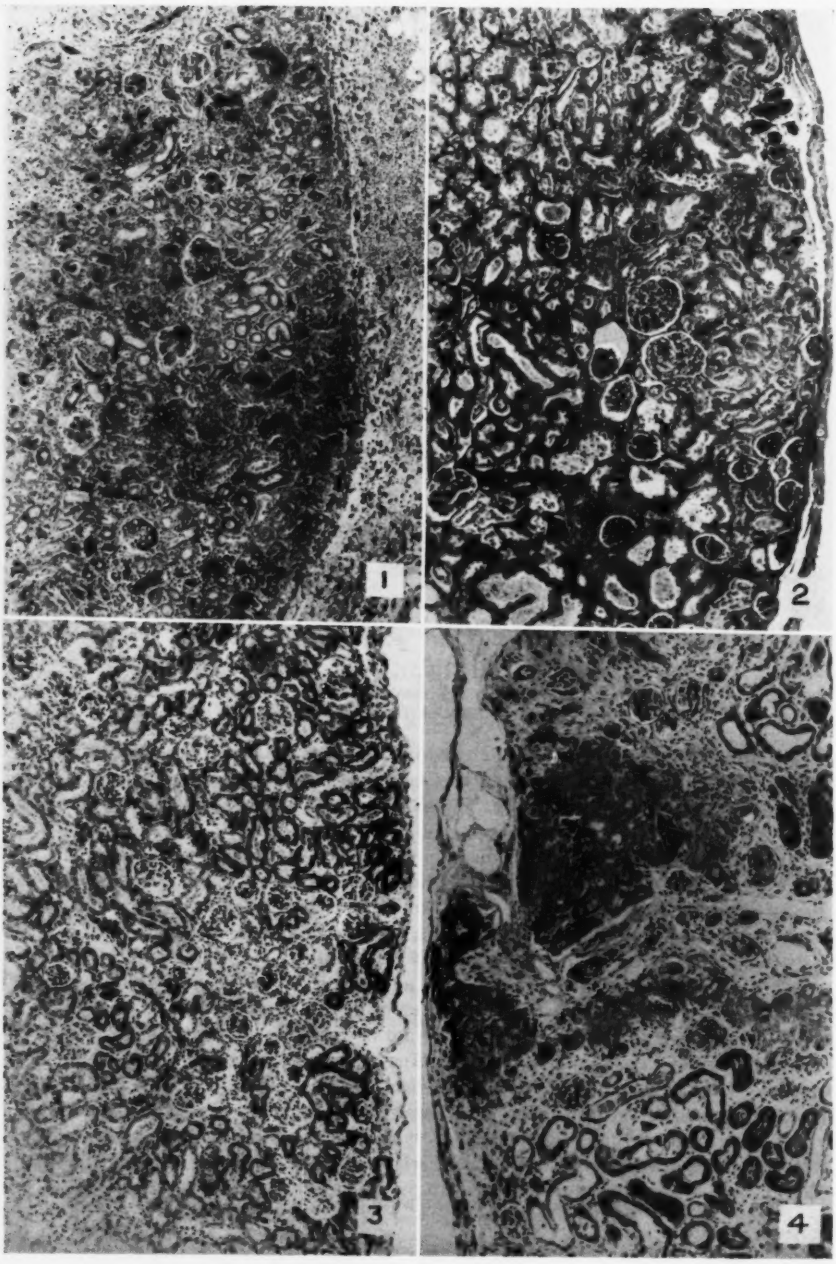

PLATE 1

mones received from 0.4 cc. to 0.5 cc. of oil per week. The rats not given hormones received no oil. Injections were begun simultaneously with the diet and were continued throughout the experiment.

All animals were weighed once per week for the first month, once per month for the following six months and at death. The rats were decapitated and exsanguinated at selected dates, or when they appeared moribund. Unfortunately, many were found dead. These were discarded from the experiment, since the tissues were unsuitable for microscopic study. Autopsies were performed, and the kidneys, liver, spleen, adrenals, pituitary, ovaries, and testes were weighed. Together with the pancreas, lungs, and salivary glands, these organs were fixed in Zenker's solution or formalin, sectioned, and stained with phloxine-methylene-blue or haematoxylin-eosin. All the organs were studied microscopically, but observations on the livers and kidneys only are reported here. Renal lesions were described and graded according to severity. The hepatic lesions were described, and the fat vacuolation and cirrhosis graded.

RESULTS

Mortality Among Young Female Rats with Severe Choline Deficiency

It has been stated above that many young female rats died during the first week on the choline-free diet. The mortality differed significantly among the groups. Two of the 18 females on diet F alone and three of the 25 receiving estradiol dipropionate died within the first 7 days of the experiment. *In the same period ten of the 24 rats receiving testosterone propionate expired.* The aggravating influence of testosterone propionate continued throughout the experiment as will be shown later.

Renal Lesion in Young Female Rats—Microscopic Details

By systematic sacrifice of rats at intervals throughout the course of the experiment, it was possible to form a composite picture of the course of the renal changes. The renal lesions in choline-deficiency of rats have been adequately described by several authors (Griffith and Wade, 1939; Gyorgy and Goldblatt, 1940; Engel and Salmon, 1941; Christensen, 1942; and Wachstein, 1944). The changes reported here correspond to those noted by other investigators.

EXPLANATION OF FIGURES

FIG. 1. Massive renal cortex damage on the seventh day of the choline-deficient diet in a rat from group FA. Hemorrhage into the capsule and between the tubules, necrosis of the epithelium, and dense casts are pictured.

FIG. 2. Renal damage, grade 1, in a rat from group FE, sacrificed at 79 days. Small clusters of atrophic glomeruli and dilated tubules lie beneath the thickened capsule.

FIG. 3. Renal damage, grade 2, in a rat from group FE, sacrificed at 149 days. Atrophy of tubules in larger foci and deeper extension into the cortex than in grade 1 lesions.

FIG. 4. Renal damage, grade 3, in a rat from group FA, sacrificed at 234 days. Large masses of acellular eosinophilic debris extend deep into the cortex. Fibrous tissue surrounds the periphery.

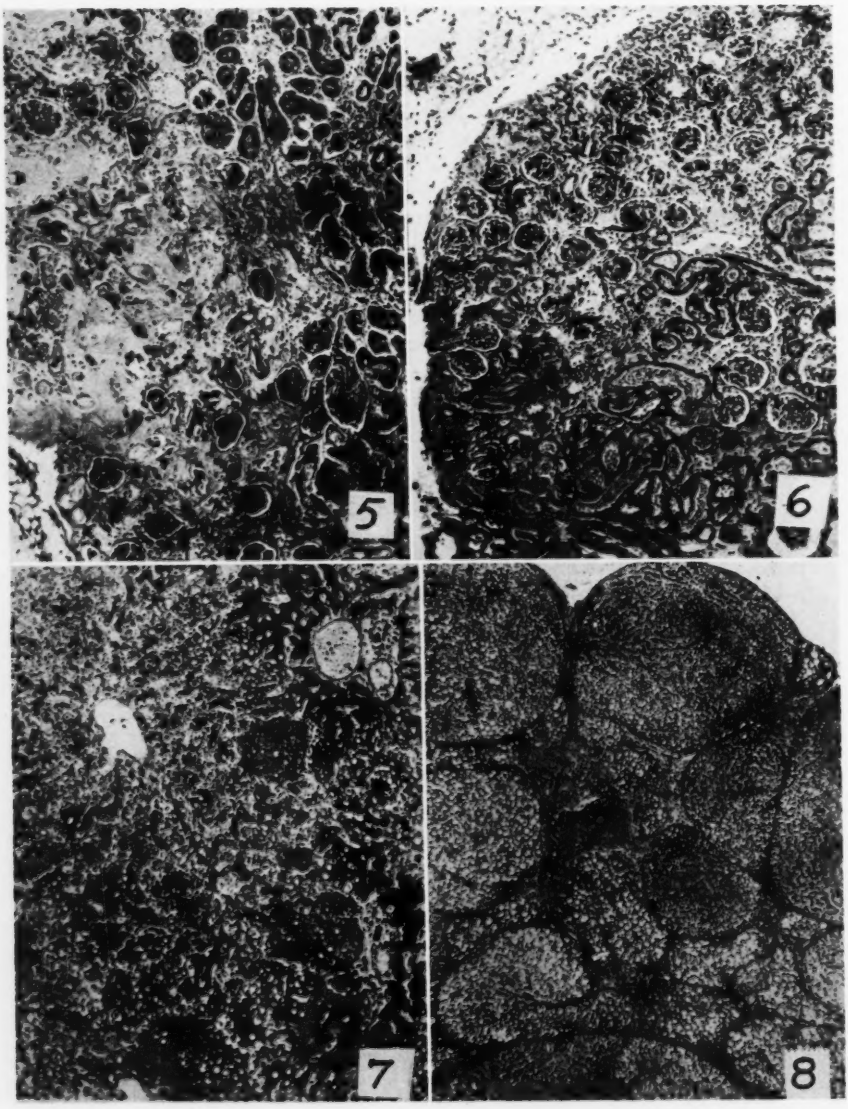

PLATE 2

During the first 7 to 8 days, massive necrosis of cortical tubules and intense hyperemia of the cortex were the chief pathological features in the young female rats (Figure 1). Total necrosis affecting all structures in large areas of the cortex appeared in the most severely affected animals. The outer half of the cortex was always more affected than the inner half, and in some instances, the necrosis was limited to the outer cortex. Usually, however, the glomeruli were spared. The renal changes found in animals that survived the necrotic phase were of a reparative nature; the emergency choline supplement apparently was sufficient to prevent further damage. The process of repair, as deduced from the specimens obtained at intervals throughout the experiment, was as follows. After the subsidence of the initial hyperemia and the removal of most of the blood pigment by the phagocytes, the necrotic portions of the parenchyma which remained were surrounded by quite normal-appearing cortical structures. In the more severe cases, large masses of necrotic material, either structureless (Figure 5) or with a suggestion of tubular shapes (Figure 4) remained. These masses were slowly filled in from the periphery by scar tissue (Figure 4). Other lesions in which scattered groups of tubules had originally degenerated, were almost completely removed by phagocytes. Only clusters of glomeruli without tubules remained. The glomeruli were approximately one-half normal size but were otherwise intact (Figures 2, 3 and 6). In several instances, dense casts failed to absorb, and many months later, they were seen in foci of dilated tubules. Finally, evidence of the original disease was demonstrated by the presence of thickened capsules, dense scars, atrophic glomeruli, a few dilated cast-filled tubules, and occasional deposits of calcium. Some arterioles in scarred areas were sclerotic; others in the rest of the parenchyma appeared normal.

METHOD OF GRADING RENAL LESIONS

An estimate of the extent of the renal damage was made for each rat, and the lesions were graded from 1 to 4. To establish a basis for comparison of the animals sacrificed at different times, the qualitative distinctions

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EXPLANATION OF FIGURES

FIG. 5. Renal damage, grade 4, in a rat from group F, sacrificed at 87 days. An almost amorphous coagulum, in which the shapes of necrotic tubules are suggested, replaces large areas of cortex.

FIG. 6. Renal damage, grade 3, in a rat from group FA, sacrificed at 234 days. An isolated wedge-shaped scar in which numerous glomeruli survive.

FIG. 7. Hepatic necrosis in a rat from group FE. Massive destruction of liver cells in the central and mid-zones on the seventh day of choline deficiency.

FIG. 8. Hepatic cirrhosis with a nodular pattern in a rat from group FA, sacrificed at 234 days.

between the lesions were ignored. If small, discrete, subcapsular foci only were present (Figure 2), the damage was designated grade 1. Somewhat deeper extension coupled with a tendency for adjacent lesions to coalesce was classified grade 2 (Figure 3). Grade 3 represented extensive involvement of most of the outer portion of the cortex, and grade 4 was similar but with deeper spread (Figures 4 and 5). Large discrete triangular scars were sometimes found with the apex reaching the cortico-medullary junction (Figure 6). These were included in grade 3.

TABLE 1. RENAL DAMAGE IN YOUNG FEMALE RATS ON A HIGH FAT, CHOLINE-DEFICIENT DIET WITH AND WITHOUT HORMONE INJECTIONS*

Group†	Number of animals	Grade of renal damage			
		0	1	2	3 or 4
F	9	1	3	3	2
FA	13	0	1	1	11
FE	6	1	2	1	2
C	10	10	0	0	0
CA	4	4	0	0	0
CE	4	4	0	0	0

* Fatalities during the first week are excluded.
† F—high fat, choline-deficient diet; A—androgen injections; E—estrogen injections; C—control stock diet.

THE EFFECT OF TESTOSTERONE PROPIONATE

Table 1 lists the effects of diet F on the kidneys of young female rats when the diet was administered from 7 to 234 days. The distribution of graded lesions and the numbers of the kidneys severely damaged (grades 3 and 4) is given for each group.

This experiment demonstrates that testosterone propionate aggravates the renal change caused by choline deficiency. The fatal renal damage that occurred in the females during the first few days of the experiment has already been discussed. Useful microscopic preparations were available in the female series for 13 of the surviving FA animals, 9 of the F animals, and 6 of the FE animals. Eleven of the 13 that received testosterone propionate had grade 3 or 4 renal disease. Only two fed the deficient diet without hormonal injections and two rats given estrogen had severe lesions (grade 3 or 4).

ABSENCE OF RENAL DAMAGE IN ADULT MALE RATS

Griffith (1940) showed that after the 30th day of life, a choline-free diet less readily produces acute renal lesions in rats. The adult males had normal kidneys after receiving diet F for 100 to 143 days. Estradiol dipropionate and testosterone propionate did not detectably alter the size or condition of these organs.

TABLE 2a. HEPATIC FAT IN YOUNG FEMALE RATS ON A HIGH FAT, CHOLINE-DEFICIENT DIET WITH AND WITHOUT HORMONE INJECTIONS*†

Group	Number of animals	Number of animals with fat vacuolation				
		0	1	2	3	4
F	10	0	1	0	3	6
FA	15	0	1	1	3	10
FE	9	0	2	3	3	1
C	10	10	0	0	0	0
CA	5	5	0	0	0	0
CE	6	6	0	0	0	0

* Fatalities during the first week are excluded.
† Duration of the dietary period was varied by systematic sacrifice.

LIVER LESIONS

The most important liver lesions in this experiment were fat vacuolation, necrosis, and cirrhosis. The lesions in these animals were similar in character to those that have been described in relation to choline-deficiency in rats by several authors (Gyorgy and Goldblatt, 1939; Lillie *et al.*, 1942; and Gyorgy, 1944). Fat accumulation appeared rapidly and persisted throughout the deficiency. Massive necrosis (Figure 7) appeared in the female series during the first 7 days, coincident with the peak of the renal disease. Some animals with extensive kidney congestion and nephrosis failed to develop necrosis of the liver, thus indicating that the kidney was the more sensitive organ. After the small emergency supplement of choline was given to the animals, the livers remained fatty. Widespread necrosis of the liver became less common and disappeared. However, one of the groups, the FE females, continued to have central necrosis in the later period as described below. Cirrhosis was not found until after the third month on diet F.

FATTY LIVERS

Liver sections of the experimental rats were graded microscopically from 0 to 4 according to the degree of fatty vacuolation of the liver cells. Unequivocal differences appeared between the groups.

TABLE 2b. HEPATIC FAT IN ADULT MALE RATS ON A HIGH FAT, CHOLINE-DEFICIENT DIET WITH AND WITHOUT HORMONE INJECTIONS FOR 4½ MONTHS

Group	Number of animals	Number of animals with fat vacuolation				
		0	1	2	3	4
F	10	0	0	0	1	9
FA	10	0	1	0	0	9
FE	10	4	2	1	3	0
C	5	0	0	0	0	0
CA	4	0	0	0	0	0
CE	4	0	0	0	0	0

Choline deficient rats receiving estradiol dipropionate had considerably less fat in their livers than those on diet F alone, or those on the diet plus testosterone propionate (Tables 2a and 2b). Six of the 10 females on diet F and 10 of the 15 on the diet plus androgen had massive (grade 4) fatty vacuolation. One of the 9 females that received estrogen had a grade 4 fatty liver. At least a few fatty vacuoles were found in all the sections of liver taken from the female rats, even those from animals "protected" by estrogen.

Suppression of the fatty vacuolation appeared also in the male rats receiving estrogen. Nine of the 10 males on the choline-free diet alone and 9 of the 10 given testosterone propionate had massively fatty livers. None of the 10 estrogen-treated males had massive fat deposits. Of the 10 males receiving estrogen, 4 had livers which appeared entirely normal. None of the others were fat-free.

The relatively low fat in the estrogen-treated rats was not due to loss of appetite with consequent failure to consume the high fat diet. The estrogen and androgen-treated animals, both those on diet F and those on the stock diet, tended to weigh less than the rats not receiving hormones, but there was little difference in the weights of the androgen and estrogen-treated groups. Moreover, there was no constant correlation between the weights of the individual animals and the degree of fatty infiltration. Small rats that might have consumed less diet than the larger animals had grade 4 fat fully as often. Individual caging and thus determination of individual food consumption was not possible in this laboratory.

In a separate series of 91 animals (unpublished data), the effects of estrogens and androgens on the deposition of hepatic fat in young male and female rats over a 10 to 30 day dietary period were studied in greater detail. In this series, the hormone injections were started 15 days prior to the initiation of the experimental diet, in order to insure exertion of maximal effect from the first day of the experimental diet and thereafter. At 10 days, there was little difference between the groups, but by 30 days, there was histological evidence of partial protection from fatty infiltration of the liver in the estrogen-treated animals. The differences were not so marked, however, as those found after a longer lapse of time as reported in Tables 2a and 2b.

CIRRHOSIS

A number of the animals of both sexes that survived 3 months or longer on the choline-deficient diet developed cirrhosis. The lesions usually were of the typical, irregularly nodular type with bands of fibrosis forming pseudo-lobules (Figure 8).

The lesions of the choline-deficient females receiving estrogen were

different from those of other animals. In contrast to the disorderly arrangement seen in nodular cirrhosis, the overall lobular pattern remained intact, and except in central area, the liver cords were normally formed. In the central zones, cells with large fat vacuoles were collected. Central necrosis appeared in many specimens. Animals sacrificed in the latter half of the experimental period demonstrated proliferating fibroblasts in the zone of necrotic liver cells. Occasional thin fibrous strands reached out to a portal area. Small branching bile ducts extended along these strands into the central regions.

Both the portal type of cirrhosis and that accompanying central necrosis were encountered by Lillie *et al.* (1942), but no correlation with the sex of the animal nor the effect of sex steroid injections was mentioned.

TABLE 3. CIRRHOSIS IN YOUNG FEMALE RATS AND ADULT MALE RATS ON A HIGH FAT, CHOLINE-DEFICIENT DIET WITH AND WITHOUT HORMONE INJECTIONS*

Group & sex	Number of animals	Grade of cirrhosis				
		0	1	2	3	4
F ♀	7	0	1	4	1	1
FE ♀	7	0	1	4	1	1
FA ♀	10	0	0	1	4	5
F ♂	10	4	5	1	0	0
FE ♂	10	10	0	0	0	0
FA ♂	10	5	1	2	2	0

* For the females, the feeding period was from 90 to 234 days; for the males, from 101 to 143 days.

"Ceroid" pigment (Lillie *et al.*, 1942) was present not only in the fibrous areas of the livers of the estrogen-treated animals but also in cirrhotic bands in animals which had received diet F alone and those given androgen.

No explanation for the unusual character of the lesions in the female rat livers was evident. Pyometrium was common in these animals. The extent to which this infection influenced the type of liver lesion was impossible to estimate. Septicemia, secondary to pyometrium, probably was not a factor, since there was no evidence of cholangitis.

THE EFFECT OF TESTOSTERONE PROPIONATE ON CIRRHOSIS

On comparison, definite variation was apparent in the amount of cirrhosis of the liver found in the animals of the different groups (Table 3). *All females surviving 3 months or longer developed cirrhosis, but testosterone had a definite aggravating effect.* Nine of the 10 females that were given testosterone propionate had grade 3 or 4 cirrhosis, whereas only 2 of the 7 animals in the estrogen-treated group and 2 of the 7 receiving no hormones had more than grade 2 cirrhosis.

The degree of cirrhosis present in the males varied. These animals,

most of which were sacrificed at about 4½ months, had signs of early cirrhosis. Six of the 10 male rats on diet F alone and 5 of the 10 on the diet plus testosterone propionate had cirrhosis, but none of those receiving estrogen had any cirrhotic lesions. Again, there was a slight but definite difference in the degree: 4 of the fat-androgen animals had cirrhosis grade 2 or 3, whereas only 1 male receiving the fat diet alone had cirrhosis as severe as grade 2.

DISCUSSION

In analogy to the present findings, it may be pointed out that Laennec's cirrhosis in the human being is predominantly a male disease. The question may be raised as to whether the male and female sex hormones play a significant role in influencing the degree of susceptibility to this disease, providing that predisposing factors are present. *The corollary question may be asked as to whether estrogen may be worth trying as an adjuvant to standard therapy used in the treatment of the early stages of portal cirrhosis in the human male.*

Since the completion of this work, Best and Hartroft (1949) have reported the occurrence of hypertension, cardiac enlargement and elevated non-protein nitrogen in association with residual renal damage several months after a brief period of acute choline deficiency. They found good correlation between the severity of the kidney lesions and the severity of the hypertension. The conditions of the experiment with young female rats reported here were similar in a number of respects, to those used by Best and Hartroft. On this basis, it seems likely that testosterone propionate might aggravate the hypertension following acute choline deficiency.

No mechanistic explanation is offered for the effect of the hormones. It is possible that the activity of choline oxidase (Handler, 1949), may be influenced or that the mobilizing action of the anterior pituitary on the deposition of fat (Stetten and Salcedo, 1944) may be altered by the hormonal injections. Whether the hormones exert their effects directly on the liver and kidney or by way of some indirect mechanism remains undecided.

SUMMARY

Testosterone propionate was found to aggravate both the renal and hepatic lesions resulting from a high fat, low choline diet in a group of young female rats and the hepatic lesions in a group of adult male rats. Administration of estradiol dipropionate appeared to afford a measure of protection against the development of cirrhosis in adult male rats and the deposition of fat in the livers of both sexes. Deaths from acute choline deficiency were more numerous for the young females receiving testosterone propionate than for those receiving no hormones or estradiol dipropionate.

Acknowledgements

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FUNCTIONAL RELATIONSHIP BETWEEN THE ADRENAL CORTEX AND POSTERIOR PITUITARY[1,2]

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IT HAS been shown that the hormones of the adrenal cortex and posterior pituitary have antagonistic actions on sodium and water excretion in suitably devised experimental conditions (Silvette and Britton, 1938a, 1938b, 1938c; Ingram and Winter, 1938; Winter *et al.*, 1938, 1939; Corey *et al.*, 1939; Silvette, 1940a, 1940b; Britton and Corey 1941; Corey *et al.*, 1941; Mulinos *et al.*, 1941; Anderson and Murlin, 1942; Britton and Kline, 1942; MacGavack *et al.*, 1942; Winter and Ingram, 1943; Little *et al.*, 1947; Roemmelt *et al.*, 1949; Sartorius and Roberts, 1949; Gaunt *et al.*, 1949; Lloyd and Lobotsky, 1950). On the other hand, hormones from both glands enhance potassium excretion (Sartorius and Roberts, 1949). The physiological significance of such facts is far from clear. One confusing factor is the inconstancy and variation of actions of the posterior pituitary hormones on electrolyte excretion and uncertainty concerning whether the same or different pituitary hormones affect water and electrolyte excretion (Heller and Stephenson, 1950; van Dyke, 1951; Anslow *et al.*, 1948; Hare *et al.*, 1943; Grollman and Woods, 1949). The variability of action of the adrenal cortical hormones is only slightly, if any, less perplexing (Gaunt, Birnie and Eversole, 1949; Woodbury *et al.*, 1950).

Whether the opposing actions of adrenal cortical and posterior pituitary hormones in sodium and water excretion manifests the operation of an integrated check-and-balance system which normally regulates the metabolism of these substances, or whether antagonism in certain respects is only an incidental manifestation of otherwise unrelated activity is an unanswered question (Shannon, 1942; Gaunt *et al.*, 1949). Silvette, Britton and Corey in proposing the idea of a functional antagonism between these two glands implied the existence of a closely coordinated type of activity. Critical evidence on the point is admittedly difficult to obtain. The

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pharmacological demonstration that antagonism occurs when the two hormones are injected simultaneously in excess of need does not provide conclusive evidence on the point. The question is, are the two types of hormones released under physiological conditions in a coordinated reciprocal manner as if designed to achieve homeostasis?

We have attempted a two-fold experimental approach to this question. First, it was reasoned that if a check-and-balance mechanism existed, the administration of a slight excess of the hormones of one gland should call forth a compensatory hypersecretion of the other.

When it was found that this probably was not the case, an attempt was made to determine whether a condition (water loading) thought to depress the rate of antidiuretic hormone (ADH) secretion would conversely increase the rate of cortical hormone secretion.

METHODS

Sprague-Dawley male rats weighing from 200 to 275 grams were used in all experiments. Depletion of adrenal ascorbic acid was used as an index of adrenal stimulation. Ascorbic acid was determined by the method of Roe and Kuether (1943). The design of the experiments followed the methods used by Sayers and associates (1948) in analogous work, and we are indebted to Dr. George Sayers for his advice concerning the techniques used.

Will an excess of either adrenal cortical or posterior pituitary hormone call forth a hypersecretion of the other?

1. *Stimulation of the Adrenal Cortex by Administered Pitressin.* Various doses of pitressin were injected intraperitoneally into rats allowed water but no food for 24 hours. At varying intervals after the treatment, the adrenals were removed and the ascorbic acid content was determined as an index of adrenal stimulation. Two groups of controls were observed simultaneously. One group received no treatment other than the fasting; the second group was injected with warmed 0.9 per cent sodium chloride solution in a volume (0.5 ml.) equal to that of the pitressin solution used.

It can be seen from Figure 1 that either 400 or 100 mU. of pitressin caused a marked stimulation of the adrenals as indicated by ascorbic acid depletion. These large doses might be regarded as "stresses" and hence the specificity of the response questioned. Of more significance was the fact that 5 mU. of pitressin had no effect on adrenal ascorbic acid. This dose is, however, one that can exert definite physiological actions (antidiuresis, chloruresis, natriuresis, etc.) as seen repeatedly at other times in this and other laboratories.

It can be concluded, therefore, that while large amounts of pitressin evoke adrenal stimulation, small doses presumably in the physiological range do not.

2. *Stimulation of the Posterior Pituitary by Administered Cortical Hormone.* This problem was not investigated directly but a small amount of data in the literature bear on the point. Birnie *et al.* (1950), in experiments designed for other purposes, reported results suggesting, but not extensive enough to prove, that an excess of either desoxycorticosterone or adrenal cortical extract might increase the antidiuretic activity of rat blood serum. Skahen and Green (1948) more conclusively showed that rats bearing desoxycorticosterone pellets excreted an antidiuretic substance in urine.

While these results suggest that an excess of cortical hormone may stimulate the posterior pituitary, this is not proved by data yet available. Further low dosage experiments are needed.

Effect of water and saline loads on adrenal ascorbic acid

It is generally accepted that an excess of water in the body depresses the rate of secretion of ADH from the posterior pituitary and that due to this fact the excess water escapes through the kidney. A large amount of work has shown, however, that such a rapid water diuresis cannot occur unless some effective amount of adrenal cortical hormone is present (Gaunt *et al.*, 1949).

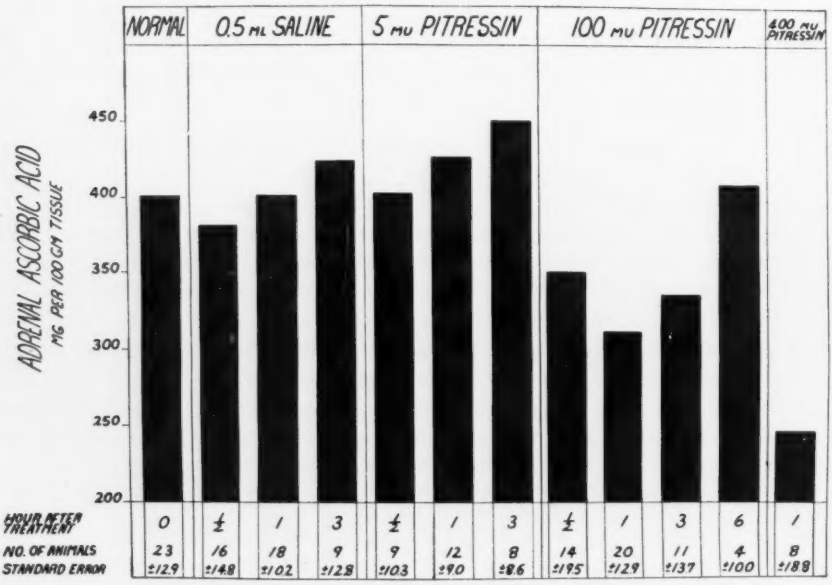

FIG. 1. Showing the adrenal ascorbic acid content at varying intervals of time after normal saline injections (a control procedure) and after various doses of pitressin.

These facts raise the question as to whether a water load is normally excreted only by depressing the secretion of ADH, or if there is in addition a simultaneous stimulation of the adrenal cortex, the hormones of which would serve to augment the diuretic response.

This question was investigated by determining whether various water loads stimulated the adrenal as indicated by the ascorbic acid depletion test. The fluid loads were administered generally in doses of 3 ml. per 100 sq. cm. of body surface warmed to body temperature. One such dose for a 220 gm. rat, for instance, was 10 ml. Since water was given by stomach tube, three groups of controls were used: (a) animals receiving no treatment, (b) animals in which a stomach tube was inserted the same number of times that water was given, but nothing introduced into the stomach, (c) animals in which air was injected into the stomach in a volume equal to that of the fluid used.

The insertion of a stomach tube or the administration of air caused only a transient fall in adrenal ascorbic acid (Figure 2), a result not increased in magnitude if the stomach-tubing was repeated three times at hour intervals. Air pockets were grossly visible in the intestine of the air-injected animals at one hour after administration.

Figure 2 shows that there was no measurable depletion of adrenal ascorbic acid when 1.5 ml. of water per 100 sq. cm. (one-half the usual dose) was given, although that was an amount sufficient to cause a substantial water diuresis. One full dose of water (ap-

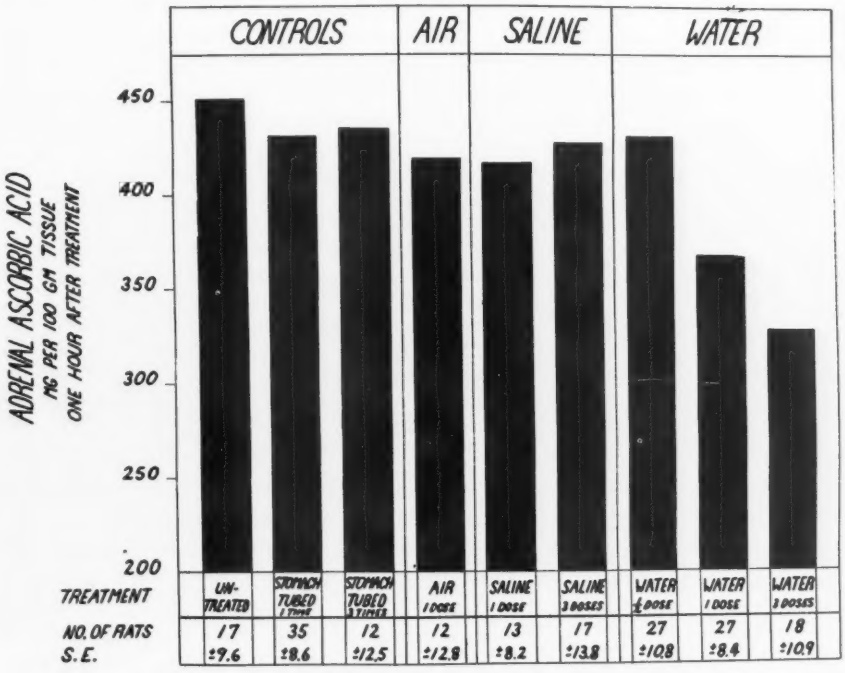

FIG. 2. Showing the adrenal ascorbic acid content after various control procedures (stomach-tubing and introduction of air into the stomach) and after the administration by stomach tube of varying amounts of 0.9 per cent sodium chloride solution or distilled water.

proximately 10 ml.), on the other hand, did cause significant reduction in adrenal ascorbic acid content, presumably indicating adrenal stimulation Three doses of water given an hour apart produced a still greater fall in adrenal ascorbic acid. In the latter experiments the adrenals were removed for analyses one hour after the last dose of water was given.

It is concluded, therefore, that while small water loads (approximately 5 ml.) fail to stimulate the adrenal cortex to increased activity, slightly larger ones will do so.

Serum sodium concentrations, kindly determined for us by Mr. Martin Ushkow, were reduced by all three water loads used. The reduction was 2.1 per cent for the half dose, 3.7 per cent for one dose and 5.2 per cent for three doses (cf. Fig. 2). These results

confirm the well-established effect of water administration on serum sodium under such conditions.

When normal saline solution was given instead of distilled water no depletion of adrenal ascorbic acid was seen with either one or three doses. Serum sodium values were not reduced by these saline treatments. It is clear then that the type of adrenal stimulation observed in water-treated animals is not a consequence of fluid load *per se*, but is due to some other factor—perhaps a reduction of osmotic pressure of plasma.

Observations on adrenal medullectomized rats

An attempt was made to determine if the effects of water and of pitressin on adrenal ascorbic acid as described above were mediated in any part through the adrenal medulla. Results were inconclusive but they are mentioned because they raise another problem worthy of further study. Various doses of water and pitressin were given to animals in which the adrenals had been enucleated and three weeks allowed for cortical regeneration. None of the treatments which reduced adrenal ascorbic acid in intact animals did so in the medullectomized ones. We do not consider the results significant because control levels of adrenal ascorbic acid were only 192 mg. per 100 gm. adrenal tissue, a value about one-half normal. Consequently, the ascorbic acid depletion test, based upon a fall from a much higher initial level of ascorbic acid, is probably invalid in such cases. The work of Gordon (1950), which appeared after our experiments were done, shows that the ascorbic acid levels do not reach their maximum and stabilize until 32 days after demedullation. Even then these levels are still below those of normal animals for some reason difficult to conjecture. The question of why the ascorbic acid content of regenerate adrenals is chronically low would be an interesting one to explore.

DISCUSSION

Previous work has shown clearly that under certain experimental conditions antagonistic actions between the hormones of the posterior pituitary and adrenal cortex can be demonstrated on water and sodium chloride excretion. The physiological implications of these facts have been reviewed elsewhere (Gaunt, Birnie and Eversole, 1949; Pitts and Sartorius, 1950).

It has not been established whether or not the hormones of these two glands are secreted under ordinary physiological conditions in a sensitively coordinated manner which helps to achieve salt and water balance. The experiments reported here show that a small but effective dose of pitressin (5 mU.) does not call forth any immediate compensatory hypersecretion of the adrenal cortex as judged by the ascorbic acid depletion test. Large doses will do so. There is some evidence, conversely, that overdoses of cortical hormone will stimulate the posterior pituitary. It does not seem likely, however, that under physiological conditions an elevated blood level of one of these hormones will, *per se*, serve as an incitant for secretion of the other.

An integrated activity of these two glands is, however, suggested by another line of evidence. Although the rapid excretion of excess body

water cannot occur without some effective amount of cortical hormone being present (Gaunt, Birnie and Eversole, 1949), our experiments indicate that the normal elimination of a mild water load (5 ml. in a 220 gm.) rat is not dependent upon an *accelerated* activity of the adrenal cortex. Larger water loads (10 ml. in a 220 gm. rat) do produce measurable stimulation of the adrenal cortex, the increased activity of which presumably serves as a stimulant to water excretion (Boss, Birnie and Gaunt, 1950). Larger doses of water provide still greater adrenal stimulation. According to current theory, a water load produces water diuresis because the induced fall in osmotic pressure of the plasma inhibits the release of posterior pituitary antidiuretic hormone (Chambers, *et al.*, 1945; Verney, 1946, 1947). We would suggest that there is an additional factor: if the water load is a sizable one, its elimination is accomplished both by an inhibition of the posterior pituitary and by a stimulation of the adrenal cortex.

The fact that even large doses of normal saline (30 ml. in a 220 gm. rat) did not cause depletion of adrenal ascorbic acid shows that the stimulus to the adrenal was not the fluid load as such. It is possible that after hydration a falling plasma osmotic pressure may both "turn off" the posterior pituitary and "turn on" the adrenal cortex, and thus by withdrawing a stimulant to the renal tubular reabsorption of water and adding an inhibitor to that process (Boss *et al.*, 1950) rapid water diuresis is insured.

It should be stated clearly, however, that these experiments provide no direct or conclusive evidence that the stimulus to the adrenal was actually a falling osmotic pressure of plasma. It could well have been some other factor associated with hydration. The assumption that osmotic changes were causal is a working hypothesis. Direct evidence on the question is being sought. It also remains to be determined whether the adrenal stimulation caused by hydration is or is not mediated through the anterior pituitary.

The work reported here is consistent with that of Danford and Danford (1950) who found that chronic water loading in rabbits leads to an increased 17-ketosteroid excretion and that there was a high positive correlation between the urine volume and the 17-ketosteroid output.

Unpublished work by Dr. Charles W. Lloyd and collaborators of Syracuse shows, on the other hand, that adrenal cholesterol levels and corticosteroid excretion may respond to fluid loads in a somewhat different manner than adrenal ascorbic acid in that the effects of large doses (roughly 30 ml. in three doses) of water and saline are similar. Be that as it may, the clean-cut results with the ascorbic acid test shows that a small amount of water evokes some sort of adrenal activity which even large doses of saline do not.

SUMMARY

1. Small doses (5 mU.) of pitressin did not stimulate the adrenal cortex as judged by the ascorbic acid depletion test in rats. Larger doses (100 or 400 mU.) did do so. It is doubtful, therefore, if a small excess of pitressin causes any direct or immediate compensatory hypersecretion of the adrenal cortex.

2. A light water load (1.5 ml. per 100 sq. cm. body surface) did not stimulate the adrenal cortex, again as judged by the ascorbic acid test. Larger loads (3 ml. per 100 sq. cm. of body surface) did stimulate the adrenals.

3. Large doses of normal saline (three times the effective water loads) did not cause adrenal ascorbic acid depletion.

4. It is suggested on the basis of this and other work that while the major endocrine factor in the elimination of a small excess of body water is a suppression of secretion of the posterior pituitary antidiuretic hormone, a larger water load causes in addition increased adrenal cortical hormone secretion which augments the diuretic response.

5. The hypothesis is stated but not proved that integration of posterior pituitary-adrenal cortical activity may be achieved in hydrated states by both glands being responsive, in a converse manner, to changes in the osmotic pressure of plasma.

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THE PARATHYROID CONTROL OF SERUM CALCIUM INDEPENDENT OF RENAL MEDIATION[1]

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THERE appears to be no general agreement with respect to the efficacy of the parathyroid hormone in the absence of the kidneys. The indications of the inessentiality of the kidneys in the parathyroid control of serum calcium (Ellsworth and Futcher, 1935; Stoerk, 1943; Monahan and Freeman, 1944) have not been fully confirmed (Tweedy *et al.*, 1937; Neufeld and Collip, 1942). On the other hand, Albright and Reifenstein (1948) are of the opinion that "the main action of the parathyroid hormone is to increase phosphate excretion in the urine." Greep (1948) in his comprehensive review has probably summed up the situation correctly and concisely in his statement that "a true hypercalcemia has never been produced in such an animal" (i.e., in a nephrectomized animal) although he emphasized the adverse biochemical changes resulting from such an operative procedure. Implicit in his statement is the uncertainty regarding the *extent* of the response elicited by the hormone in the absence of the kidneys.

In the present successful attempt to measure quantitatively the parathyroid control of serum calcium in the absence of the kidneys, there were four main considerations. (1) The activity of the parathyroid was assessed, where feasible, in terms of its ability to elevate an abnormally low serum calcium level, rather than by its ability to overload with calcium a normal serum, since it is a generally accepted view that relatively smaller amounts of a hormone are required to maintain a normal homeostasis than are needed to elicit overdosage phenomena. (2) In those instances in which nephrectomized animals were utilized, the experiments were designed in such a manner as to minimize the duration of the observational period following the operation, thereby lessening, or even eliminating, the undesirable and complicating sequelae of nephrectomy. (3) Measurements on thyroparathyroidectomized animals were made at time intervals optimal with respect to residual hormone activity and the onset of thyroprival symptoms. Studies by Hastings and Huggins (1933) have

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indicated that there is still residual hormone activity six hours after removal of the parathyroids whereas there is probably none twenty-four hours after the operation. On the other hand, too long an interval may lead to interpretations confused by the symptoms consequent to thyroidectomy. (4) In order to avoid the additional variable of differential rates of absorption, or of destruction, the hormone was injected intravenously. This consideration might be of major importance when the recipient animal possesses a reticuloendothelial system rendered hyperactive by infection or by operative procedures.

METHODS

Overnight-fasted dogs of both sexes and weighing 5 to 15 kg. were employed. All surgical operations were performed with the animal under sodium pentothal anesthesia. This anesthetic was also used throughout the experimental period to facilitate intravenous injections and the collection of blood samples from the exposed jugular vein. Thyroparathyroidectomized animals were used 1 to 5 days following the operation unless otherwise stated. Nephrectomized animals, with one exception, received their experimental injections 7 to 22 minutes following ablation of the kidneys. The parathyroid extract (Lilly) contained 100 U.S.P. Parathyroid Units per ml. and was injected intravenously. Sodium oxalate was employed for the purpose of lowering the serum calcium at a dosage of 40 mg. per kg. of body weight. This was the dose reported by Patt and co-workers (1942; 1942a), but the mode of administration differed from theirs in that the oxalate was injected at the rate of 5 ml. per minute in the form of an 0.1 M aqueous solution. Serum calcium was determined in duplicate, at first by the method of Clark and Collip (1925) but early in the investigation the colorimetric method of Loureiro and Janz (1944) was adopted.

RESULTS AND DISCUSSION

The control of the level of serum calcium in nephrectomized animals was measured in two distinctly different ways: (1) by the response to the intravenously injected hormone; (2) by a study of the oxalate tolerance.

(1) *Response to parathyroid hormone*

No difficulty was encountered in demonstrating the hypercalcemic action of parathyroid extracts in the nephrectomized but otherwise normal dog. In Figure 1 are shown graphically the average serum calcium levels of 3 nephrectomized animals (N–H) following their injection with 5 ml. of parathyroid extract each, in comparison with the levels obtained when no extract was injected (N). These results fully confirm those of Ellsworth and Futcher (1935) who obtained an average increase of 3.5 mg.% (30%) above starting levels in the serum calcium of 3 nephrectomized dogs receiving a subcutaneous injection of 700 units of parathyroid hormone each. This response was but one-half that expected for normal animals on

the basis of the assayed value of the extract. In the present experiment the response calculated for the normal dog without nephrectomy was an increase of 2.5 mg.% of serum calcium, since the hormone has but one-half the activity when administered intravenously (Thomson and Collip, 1932). The obtained response in the absence of the kidneys was 2.9 mg.% (29%) increase above initial levels. The conclusion follows that the hypercalcemic action of the parathyroid hormone was unaffected by the absence of the kidneys.

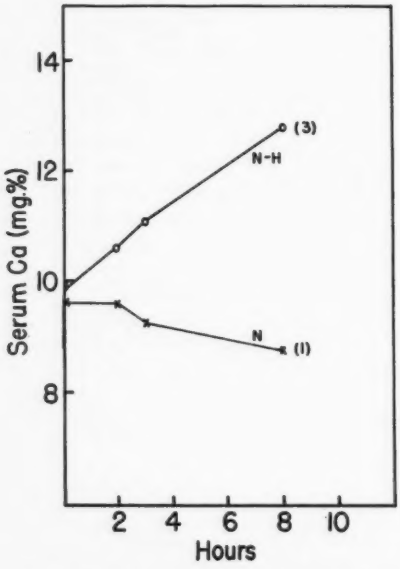

FIG. 1. Average increase in serum calcium of 3 nephrectomized dogs (N-H) following their administration with 5 ml. parathyroid extract each. N: 1 nephrectomized dog without extract.

An extension of this study to the parathyroidectomized animal yielded essentially similar results (Figure 2). Dog A was thyroparathyroidectomized immediately prior to the experiment, whereas dogs B and C were similarly operated on 1 and 5 days, respectively, preceding the experiment. Dogs A and C each received 5 ml. of the parathyroid extract while dog B was given 10 ml. It is not certain that peak values for serum calcium were obtained in these experiments; however, the data are sufficient to indicate that parathyroid extracts are capable of producing replacement therapy and overdosage phenomena independent of renal mediation. No significance is attached to the decrease in the serum calcium of the 2

control animals, (N) in Figure 1 and (D) in Figure 2. The observation is an interesting one in view of the fact that Ellsworth and Futcher (1935) reported a slight increase in the serum calcium of their 3 nephrectomized control dogs. Their results are difficult of interpretation since shock is reported to have no effect on serum calcium in the dog (Gregersen, 1946) and a *decreased* serum calcium is usually considered to be the sequel of uremia (Bradley, 1948) and of acute urinary suppression (Stock, 1949).

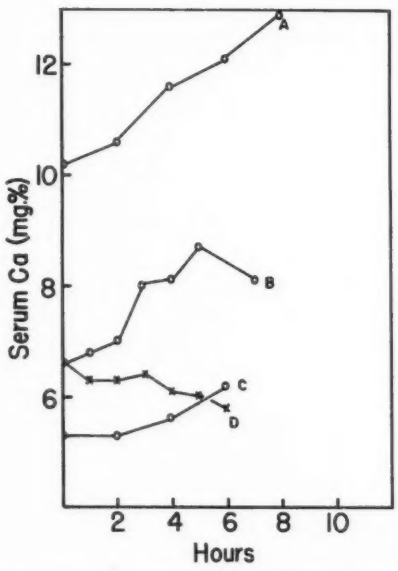

FIG. 2. Serum calcium levels of thyroparathyroidectomized-nephrectomized dogs A, B and C following their injection with 5, 10 and 5 ml. respectively of parathyroid extract. D: Thyroparathyroidectomized-nephrectomized dog without extract.

(2) *Oxalate tolerance studies*

Patt and co-workers (1942, 1942a) have shown that a characteristic curve of serum calcium concentration plotted as a function of time is obtained following the intravenous injection of a dog with a massive dose of sodium oxalate. They found that the serum calcium of a nonoperated dog returned to normal limits within a few hours following the administration of the oxalate whereas in the parathyroidectomized animal the extent of recovery was only 1 to 2 mg. per cent. They also presented strong evidence that the stimulus for parathyroid hormone secretion was a low

serum calcium level. Thus, the *oxalate tolerance curve*,[2] as a measure of parathyroid function, might be considered analogous to the glucose tolerance curve for pancreatic islet cell function in that each gives an index of an endocrine function by the ability of the respective hormone to normalize a strained homeostatic mechanism.

The data in Figure 3, compared with those previously reported, are in part confirmatory and in part at variance. The tolerance curve for the control animals (C) is intrinsically the same; however, that of the para-

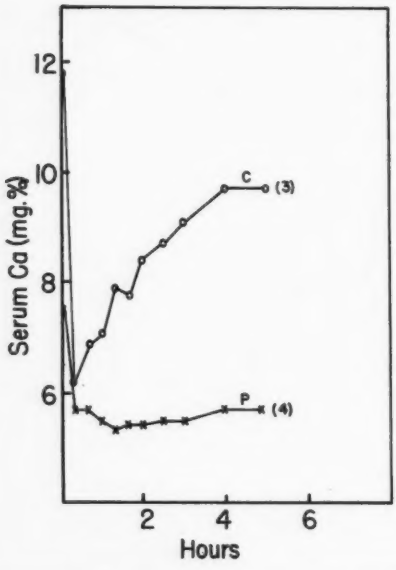

FIG. 3. Oxalate tolerance curves. C: Average curve for 3 normal control dogs. P: Average curve for 4 thyroparathyroidectomized dogs.

thyroidectomized animals (P) manifests no appreciable recovery toward normal limits. Patt and his associates considered the possibility that the partial recovery exhibited by their parathyroidectomized animals resulted from the release of calcium from the tissue fluids, from bone salts, or by partial dissolution of the formed calcium oxalate, with or without renal excretion of the oxalate. Since their oxalate injections were made immediately subsequent to parathyroidectomy, there is an alternative explanation for their observations. The partial recovery of their parathyroidectomized animals can be attributed to residual hormone activity because (a) the results of Hastings and Huggins (1933) indicated that residual hormone

[2] This designation has been suggested by the present writers.

activity persists for several hours following removal of the parathyroids; (b) the parathyroidectomized animals, (P) in Figure 3, employed in the present study failed to exhibit any recovery phase following their injection with oxalate at a time at which there was a complete lack of residual hormone activity as evidenced by the results of Hastings and Huggins.

The oxalate tolerance of the nephrectomized animal was next examined. As shown in Figure 4, the recovery phase of the nephrectomized animal

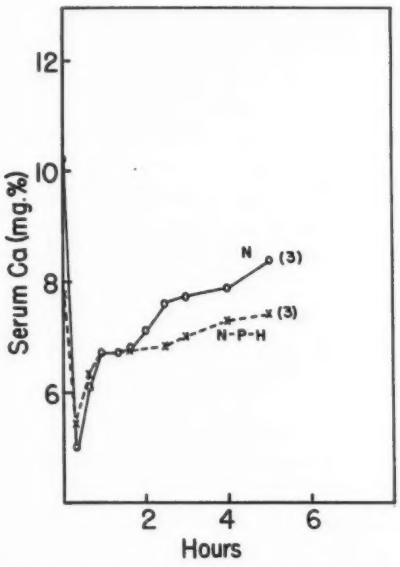

FIG. 4. Oxalate tolerance curves. N: Average curve for 3 nephrectomized dogs. N-P-H: Average curve for 3 nephrectomized-thyroparathyroidectomized dogs pretreated with 10 ml. parathyroid extract each.

(N) was virtually identical with that of the normal animal, (C) in Figure 3. Accepting the current view that the recovery phase is a function of the activity of the parathyroid, it is concluded that the parathyroid response of the nephrectomized dog was the same as that of the normal dog.

This conclusion is confirmed by oxalate tolerance studies on the thyroparathyroidectomized-nephrectomized dog (N-P-H in Figure 4) in which the function of the parathyroid glands was replaced by the administration of 10 ml. of parathyroid extract within the hour prior to the oxalate injection. Slight differences in the curves are attributed to variations in effective hormone concentration since there was present a considerable degree of overlapping of the individual components making up the two curves.

Elimination of the recovery phase by thyroparathyroidectomy, (P) in Figure 3, and its reconstitution by the administration of parathyroid hormone to the parathyroidectomized-nephrectomized animal, (N-P-H) in Figure 4, indicates that the recovery phase reflects parathyroid activity; furthermore, the conclusion that this parathyroid activity is independent of renal mediation receives additional substantiation. These data also serve to validate the explanation given above for the partial recovery exhibited by the parathyroidectomized animals employed by Patt and his associates; in addition they relegate the role of the thyroid in the present study to a position of minor importance.

The dependence of the shape of the oxalate tolerance curve on the time at which the blood samples are taken should be emphasized. This is exemplified by an experiment carried out on a nephrectomized dog from which the first 3 blood samples following oxalate administration were collected at 7, 14 and 20 minutes, respectively. The 7 minute sample showed a serum calcium of 5.5 mg.% (initial level was 10.0 mg.%) whereas the 20 minute sample revealed an increase to 6.9 mg. %. For comparative studies the necessity of arbitrary but definite time intervals is therefore stressed.

All the present data support the view that the parathyroid hormone regulates the level of serum calcium in the absence of the kidneys.

SUMMARY

Parathyroid gland extract produced within 8 hours the normal response of hypercalcemia when administered intravenously to 3 nephrectomized dogs immediately following the operation. Significant increases in blood calcium also resulted from the intravenous injection of parathyroid extract into 3 nephrectomized-thyroparathyroidectomized dogs.

The intravenous administration of sodium oxalate (40 mg. per kg.) produced in normal dogs a precipitous drop in serum calcium followed by a gradual return toward normal levels within 6 hours. That this *oxalate tolerance curve* is a measure of parathyroid function was evidenced by the fact that while a sharp drop in serum calcium was similarly produced in thyroparathyroidectomized animals, there was no appreciable return of the serum calcium toward preinjection levels.

The recovery phase of the oxalate tolerance curve of nephrectomized dogs was found to be identical to that of normal animals. In addition, nephrectomized-thyroparathyroidectomized animals manifested a normal type of curve when pretreated with parathyroid extracts.

The cumulated data support the view that the parathyroid gland exerts over serum calcium a control that, both in degree and in direction, is primarily independent of the kidneys.

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THE INFLUENCE OF CASTRATION ON FASTING METABOLISM[1]

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AS AN extension of investigations of the endocrine control of fasting metabolism (White and Dougherty, 1947; Szego and White, 1949; White, Hoberman and Szego, 1949), a study has been conducted of the role of the gonads in the mobilization of tissue constituents during fasting. This approach appeared of interest in view of the implication of androgenic hormones in net protein anabolism (cf., Kochakian and Murlin, 1935; Kenyon *et al.*, 1944; Kochakian, 1946; White, 1948) together with the observation that it was possible to demonstrate relative protein retention under the influence of growth hormone during total inanition (Szego and White, 1949). Moreover, since it appears that gonadal hormones exert an influence upon the size and enzymic activity of specific organs (cf. Korenchevsky, 1939; Korenchevsky and Ross, 1940; Korenchevsky and Hall, 1941; Ross and Korenchevsky, 1941; Kochakian, 1946), it was of interest to localize further, if possible, certain of the metabolic influences of gonadal hormones. Accordingly, in order to establish a baseline for studies of the effects of exogenous androgen administration, the present experiments were designed to examine the influence of castration on the changes in composition of two relatively labile structures, liver and lymphoid tissue, during fasting.

The data which have been obtained reveal that castration in either male or female mice results in profound alterations of lipid and water metabolism in the liver before and during fasting. The rate of protein mobilization from the liver during fasting is apparently unaffected. The present studies indicate further that the degree of involution of lymphoid tissue, which normally accompanies total inanition, is inhibited markedly in the castrate animal.

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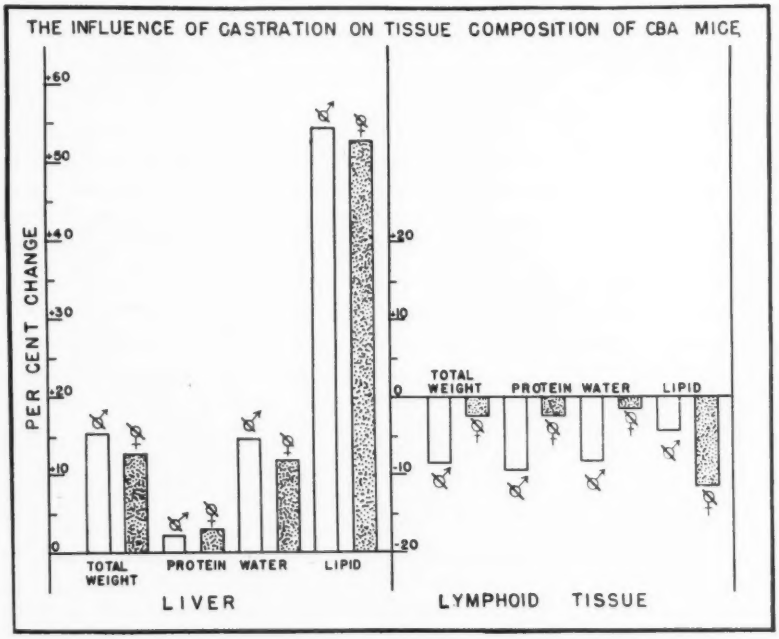

FIG. 1. In this chart, the total weight, and the weight of individual constituents of liver and lymphoid tissue per 100 gm. body weight in fed intact controls, male and female respectively, are given as the zero baseline. Changes from this baseline in castrate animals are represented by the bars. The numbers of animals contributing to the average values used in the calculations are indicated in Table 1. Values above the baseline indicate percentage increases; values below, decreases.

METHODS

Male and female mice of the CBA strain (Strong) were used. Half of each group was bilaterally gonadectomized at the age of 5 weeks. All animals were maintained on Rockland laboratory chow pellets supplemented with lettuce biweekly until they reached the age of 8 to 9 weeks and the weight range of 18 to 22 grams. At this time, one-half of the operated animals were transferred to individual metabolism cages and fasted for 48 hours with free access to water, while the remaining mice served as fed controls. At the end of the fasting period, all mice were sacrificed, and samples of lymphoid tissue and liver were obtained for the determination of water, total nitrogen and total lipid by methods which have been described (Szego and White, 1949).

Two experiments were performed on Sprague-Dawley male rats. Half of each group was bilaterally orchidectomized at the age of approximately 2 months and permitted to recover for a period of 3 weeks under conditions described above for the CBA mice. At the end of this period, half of each group (orchidectomized and non-operated) was fasted for 96 hours in individual cages with free access to water. Autopsy procedure involved exsanguination under nembutal anesthesia, determination of wet weights of

spleen, thymus, mesenteric lymph nodes, and sampling of these tissues for total nitrogen. Wet weights of adrenals and pituitaries were also recorded.

Total nitrogen multiplied by the factor 6.25 was used as an approximation of the protein content of these tissues. It is recognized that this method of calculation does not give unequivocal values for protein.

The lipid values expressed in the tables and figures were obtained by difference, utilizing the observed concentrations of water and nitrogen. These lipid figures were checked in a number of instances by direct determination of total lipid content by a gravimetric method described previously (Szego and White, 1949). The maximum percentage difference between these two methods of estimation of total lipid was found to be 5 per cent in the case of liver, and negligible with respect to lymphoid tissue, as demonstrated in an earlier report (Szego and White, 1949).

RESULTS

1. *The influence of castration on liver and lymphoid tissue composition in mice.*

Both intact and castrate mice were sacrificed in the fed state to provide data from which was inferred the tissue composition at the start of the fast. These data are illustrated in Figure 1.

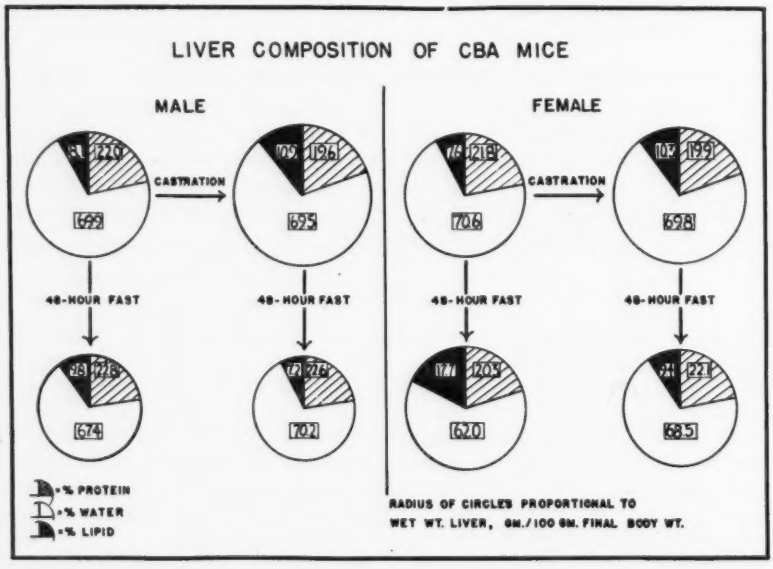

FIG. 2. A diagrammatic representation of the effects of castration and fasting on liver composition. The radius of each circle is proportional to the average wet weight of livers of the experimental and control animals per 100 gm. final body weight. The percentage distribution of the three principal tissue constituents is represented by the area of appropriately designated sectors of the resulting circles and the numbers therein.

It is apparent that 3 weeks after gonadectomy, the livers of both male and female castrates were significantly larger than those of the appropriate intact controls, which form the zero baselines for comparison. The composition of the increase was primarily water and lipid. The increase in liver water was probably related to the increase in tissue mass, whereas the lipid deposition was disproportionately larger, and occurred to a similar extent in castrates of both sexes, apparently at the expense of protein. The latter constituent was elevated slightly because of the larger liver size, but disproportionately less in relation to lipid and water.

It is of interest to note that the increase in liver lipid of fed castrates (as revealed by Figures 1 and 2 and also by Table 1) was not accompanied, as is usually the case, by a concomitant decline in relative water content (cf. Szego and White, 1949). Table 1 reveals that the increase in relative liver weight, together with the deposition of water and lipid, were statistically significant effects of castration in both sexes. The accompanying decline in nitrogen concentration under the same conditions was also statistically significant. Due to the increase in total liver mass, however, the diminished nitrogen concentration was not reflected in a significant decline in total liver nitrogen as related to body weight.

The demonstrated influence of castration on initial liver mass and composition was taken into account in all comparisons of analytical data derived from fasting animals: intact fasted mice were compared with intact fed animals, while the castrate fasted animals were compared with castrate fed mice as controls. Controls were further distinguished by sex. This basis of comparison was utilized and discussed in an earlier report (Szego and White, 1949).

In contrast to the effect of castration on liver composition, Figure 1 illustrates that the relative mass and composition of lymphoid tissue of fed castrates did not differ significantly from the intact fed controls. Similar conclusions may be inferred from Table 3. Control baselines for lymphoid tissue were nevertheless utilized as described above in the case of liver data.

2. *The influence of castration on the mobilization of liver constituents in fasting mice.*

The well-known mobilization of lipid to the liver of intact male and female animals during fasting (cf. MacKay and Barnes, 1937) is confirmed in the present studies (cf. Figure 2). This phenomenon was particularly striking in the case of the female, and served to maintain relative liver size constant during fasting. In the castrates, on the other hand, liver lipid was depleted at a faster rate than could be overcome by replenishment from the depots.

TABLE 1. THE INFLUENCE OF CASTRATION ON LIVER COMPOSITION OF FED AND FASTING CBA MICE*

Group	No. of mice	Body weight		Liver wet wt. gm./100 gm. B.W.[2]	Liver nitrogen		Liver water		Liver lipid[3]	
		Initial[1]	Final[2]		%	gm./100 gm. B.W.[2]	%	gm./100 gm. B.W.[2]	%	gm./100 gm. B.W.[2]
Intact ♂, Fed	17	—	22.2 ±0.3	6.27 ±0.12	3.52 ±0.03	0.221 ±0.004	69.9 ±0.3	4.39 ±0.08	8.1	0.508
Intact ♂, Fasted 48 hrs.	15	21.8 ±0.4	16.9 ±0.3	5.15 ±0.11	3.64 ±0.05	0.187 ±0.003	67.4 ±0.5	3.48 ±0.07	9.8	0.505
Castrate ♂, Fed	15	—	21.4 ±0.5	7.23 ±0.16	3.14 ±0.11	0.226 ±0.005	69.5 ±0.3	5.04 ±0.10	10.9	0.786
Castrate ♂, Fasted 48 hrs.	14	20.8 ±0.4	15.9 ±0.4	5.22 ±0.18	3.62 ±0.03	0.189 ±0.008	70.2 ±0.6	3.65 ±0.11	7.2	0.375
Intact ♀, Fed	9	—	18.7 ±0.6	6.08 ±0.15	3.49 ±0.04	0.211 ±0.005	70.6 ±0.4	4.29 ±0.10	7.6	0.462
Intact ♀, Fasted 48 hrs.	9	19.1 ±0.5	14.3 ±0.2	6.06 ±0.22	3.25 ±0.08	0.197 ±0.007	62.0 ±1.5	3.75 ±0.13	17.7	1.075
Castrate ♀, Fed	11	—	20.5 ±0.4	6.86 ±0.19	3.18 ±0.09	0.218 ±0.008	69.8 ±0.3	4.79 ±0.13	10.3	0.706
Castrate ♀, Fasted 48 hrs.	12	20.4 ±0.7	15.0 ±0.6	5.90 ±0.12	3.53 ±0.06	0.208 ±0.004	68.5 ±1.2	4.04 ±0.10	9.4	0.555

* Figures shown are means and their standard errors. Differences between means are considered statistically significant when they exceed by at least 3-fold the standard error of the difference, calculated by the formula:

$$\text{S.E.D.} = \sqrt{(\text{S.E.}_1)^2 + 5.\ \text{gm.}\ (\text{S.E.}_2)^2}.$$

[1] Body weight at start of 48-hour fast.
[2] Body weight at autopsy.
[3] By difference.

The profound aberrations in lipid mobilization which occur during fasting in the absence of the gonads are also indicated in Table 2. A striking net depletion of liver lipid occurred in castrate males and females during a 48 hour fast. Intact fasted males, in contrast, showed a less severe degree of liver lipid loss, while the unoperated, fasted females were capable of mobilization of lipid to the extent that a marked gain in liver lipid was observed in these animals. As a consequence, the effects of fasting on liver lipid metabolism are more dramatically influenced in the female by castration. The extra losses of liver lipid as a result of fasting in castrates

TABLE 2. THE INFLUENCE OF CASTRATION ON THE NET CHANGES IN LIVER CONSTITUENTS RESULTING FROM FASTING*

Group	Liver wet wt., gm./100 gm. B.W.‡	Liver nitrogen		Liver water		Liver lipid	
		%	gm./100 gm. B.W.‡	%	gm./100 gm. B.W.‡	%	gm./100 gm. B.W.‡
Intact ♂	−1.12±0.16†	+0.12±0.06	−0.034±0.005†	−2.5±0.6†	−0.91±0.11†	+ 1.7	−0.003
Castrate ♂	−2.01±0.24†	+0.48±0.12†	−0.037±0.009†	+0.7±0.7	−1.39±0.15†	− 3.7	−0.411
Intact ♀	−0.02±0.27	−0.24±0.09	−0.014±0.009	−8.6±1.5†	−0.54±0.16†	+10.1	+0.613
Castrate ♀	−0.96±0.22†	+0.35±0.11†	−0.010±0.009	−1.3±1.2	−0.75±0.16†	− 0.9	−0.151

* All animals had been fasted for 48 hours preceding autopsy. Figures shown are the differences between means of experimental groups and of their appropriate fed controls (see text and Table 1), and the standard errors of the differences.

† Differences are considered statistically significant when they exceed by at least 3-fold the standard error of the difference.

‡ Body weight at autopsy.

of both sexes, when calculated on a percentage basis (see legend), are illustrated in Figure 3.

Notwithstanding the peculiar inability of castrates to mobilize depot lipid in pace with liver lipid depletion during fasting, there is apparently no effect of gonadal hormone deprivation under these conditions on the rate of mobilization of protein from the liver. Table 2 reveals that the losses of liver nitrogen on a body weight basis are similar for intact and castrate members of each sex. This is further illustrated in Figure 3, which depicts similar liver protein losses in all groups during a 48 hour fast.

Loss of water from the liver in intact mice during fasting was surpassed by the rate of depletion of this liver constituent in castrate animals. Examination of the ratio of water:protein loss on a weight basis from the liver during a 48-hour fast reveals a consistently greater rate of water loss from the livers of castrate animals, *viz.*, intact males, 3.6:1, castrate males, 4.4:1; intact females, 3.7:1; castrate females, 4.5:1. This greater degree of loss of liver water during fasting in the gonadectomized groups may be related to the significant retention of liver water (cf., Table 1) which occurred despite active lipid deposition in similar fed castrate controls.

Accelerated water and lipid depletion of the livers of castrates during

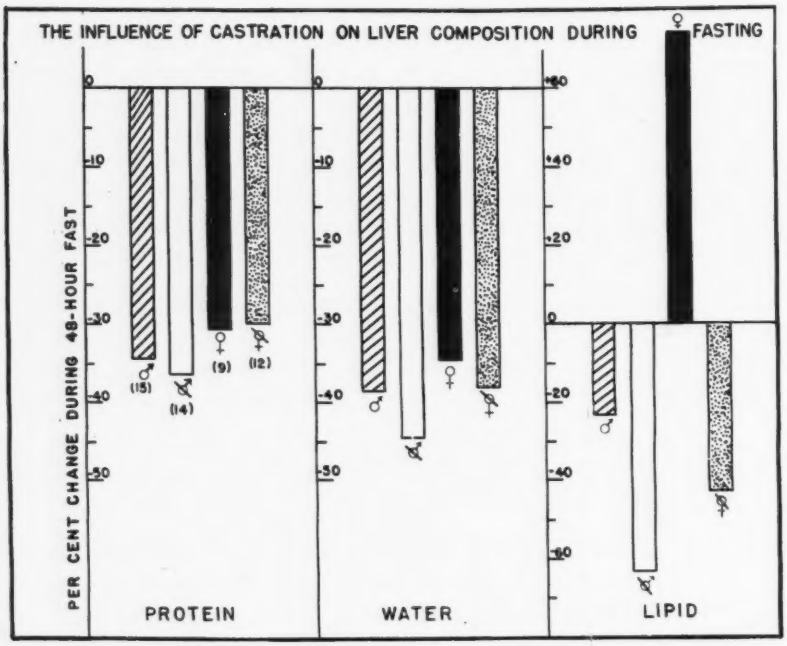

FIG. 3. The length of the bars represents per cent change in liver constituents of fasted mice related to the tissue composition of appropriate fed controls, taken as a zero baseline. Comparison is made between intact fasted *vs.* intact fed, castrate fasted *vs.* castrate fed, animals, male and female, respectively. The absolute amounts of each constituent present at the start of the fast are inferred for each group from the liver weight:body weight ratio and the liver composition found by analysis of fed controls. The fasting groups represented by the bars are indicated by the appropriate symbols. The number of animals contributing to the average in each case is indicated by the number enclosed in parentheses. Negative values indicate losses; positive values, gains.

fasting was reflected in a more rapid decline in the ratio of total liver wet weight per 100 grams body weight as compared with fasted intact controls (Table 2; Figure 2). The data indicate that the drain on liver constituents, except protein, during fasting is not as well compensated in gonadectomized as in intact animals.

3. *The influence of castration on the alterations in lymphoid tissue constituents during fasting.*

Table 3 presents data for the relative weight and composition of a constant and representative fraction of lymphoid tissue of fed and fasted castrate and intact mice. As noted earlier (cf., section 1), no significant

TABLE 3. THE INFLUENCE OF CASTRATION ON LYMPHOID TISSUE COMPOSITION OF FED AND FASTING CBA MICE*

Group	No. of mice	Body weight		Wet wt. lymphoid tissue, gm./100 gm. B.W.‡	Lymphoid tissue nitrogen		Lymphoid tissue water %	Lymphoid tissue lipid§ %
		Initial†	Final‡		%	mgm./100 gm. B.W.‡		
Intact ♂, Fed	13	—	22.4±0.3	0.885±0.027	2.70±0.05	24.1±0.9	78.5±0.2	4.6
Intact ♂, Fasted 48 hrs.	11	21.8±0.4	16.6±0.3	0.555±0.042	2.75±0.04	15.1±1.2	78.2±0.3	4.6
Castrate ♂, Fed	11	—	21.6±0.7	0.811±0.089	2.67±0.03	21.8±2.5	78.5±0.1	4.8
Castrate ♂, Fasted 48 hrs.	15	20.8±0.4	15.9±0.4	0.703±0.035	2.71±0.03	19.0±1.0	77.8±0.2	5.3
Intact ♀, Fed	9	—	18.7±0.6	1.017±0.026	2.69±0.04	27.2±2.6	78.0±0.2	5.2
Intact ♀, Fasted 48 hrs.	9	19.1±0.5	14.3±0.2	0.769±0.044	2.72±0.01	21.3±1.1	79.2±0.3	3.8
Castrate ♀, Fed	11	—	20.5±0.4	0.994±0.047	2.66±0.02	26.6±1.4	78.7±0.1	4.7
Castrate ♀, Fasted 48 hrs.	12	20.4±0.7	15.0±0.6	0.911±0.036	2.74±0.06	24.9±1.1	79.3±0.3	3.6

* Figures shown are means and their standard errors.
† Body weight at start of 48-hour fast.
‡ Body weight at autopsy.
§ By difference.

effect of castration on lymphoid tissue weight and composition was observed in fed animals 3 weeks after gonadectomy. It is of interest to note that the relatively larger lymphoid tissue weight of the fed, intact female, as compared with similar males, persists even following castration.

Castrated, fasting animals, on the other hand, showed differences in the rate of lymphoid tissue involution when comparisons were made with unoperated, fasting controls. The data in Table 4 confirm the earlier observation of the profound involution of lymphoid tissue which occurs in fasting animals (White and Dougherty, 1947). This is reflected in the highly significant decline in the wet weight of lymphoid tissue per 100 gm. of body weight as compared with this ratio in the controls. This decrease is a measure of the selective loss of lymphoid tissue protoplasm in excess of the rate of loss of total body weight. It would appear that lymphoid tissue

TABLE 4. THE INFLUENCE OF CASTRATION ON THE NET CHANGES IN LYMPHOID TISSUE CONSTITUENTS RESULTING FROM FASTING*

Group	Lymphoid tissue wet wt., gm./100 gm. B.W.§	Lymphoid tissue nitrogen		Lymphoid tissue water %	Lymphoid tissue lipid %
		%	mgm./100 gm. B.W.§		
Intact ♂	−0.330±0.050†	+0.05±0.06	−9.0±1.5†	−0.3±0.4	0
Castrate ♂	−0.108±0.095	+0.04±0.03	−2.8±2.7	−0.7±0.2†	+0.5
Intact ♀	−0.248±0.051†	+0.03±0.04	−5.9±2.8‡	+1.2±0.4	−1.4
Castrate ♀	−0.083±0.060	+0.08±0.06	−1.7±1.8	+0.6±0.3	−1.1

* All animals had been fasted for 48 hours preceding autopsy. Figures shown are the differences between means of experimental groups and of their appropriate fed controls (see text and Table 3), and the standard error of the differences.
† Differences are considered statistically significant when they exceed by at least 3-fold the standard error of the difference.
‡ Of limited significance: "p" value lies between 0.02 and 0.05.
§ Body weight at autopsy.

involution in the intact female during fasting is significantly less than in the male.

In castrate mice, as seen from the data in Table 4, there is a significantly reduced degree of involution of lymphoid tissue during fasting. There is no statistically significant difference in the ratio of lymphoid tissue weight per 100 gm. of body weight between fed or fasted castrates. It is apparent, therefore, that while mobilization of lymphoid tissue constituents in parallel with body weight losses does occur in castrate animals,

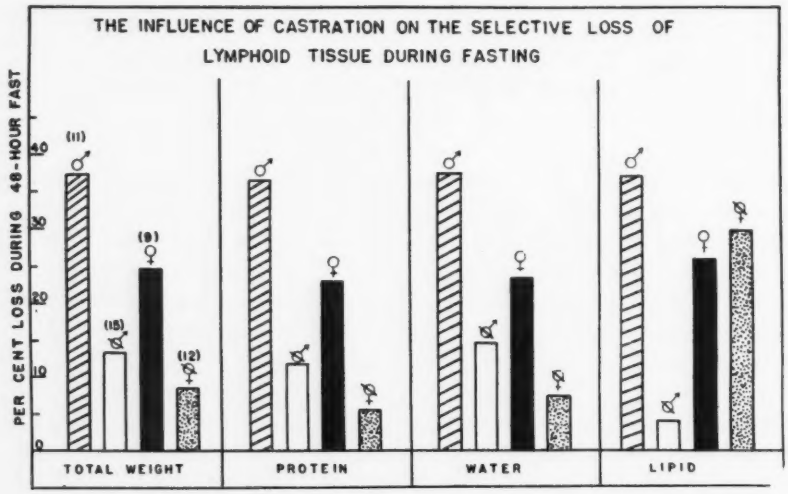

FIG. 4. This chart shows the selective loss of lymphoid tissue constituents in excess of the loss which would have been expected on the basis of body weight decline. Thus, the length of the bars represents the percentage difference between the initial and final amounts of each constituent per 100 gm. body weight at the time of sampling. The initial levels were obtained by analysis of fed controls. The average selective loss is shown for intact and castrate male and female mice designated by the appropriate symbols above the bar. The number of animals contributing to each average is indicated in parentheses below these symbols.

the significant, selective involution (in excess of the rate of body weight loss) caused by fasting in intact controls is not seen in the absence of the gonads. These data are illustrated graphically in Figure 4.

The composition of the absolute decrement in lymphoid tissue weight during fasting does not appear to differ significantly in castrate, as compared to intact, animals except in the case of lipid in the gonadectomized females. In the latter group, loss of this constituent exceeds the rate of reduction of lymphoid tissue protein and water. This relative endocrine

independence of the rate of mobilization of lymphoid tissue lipid has been noted earlier (Szego and White, 1949).

The influence of castration in retarding the involution of lymphoid tissue during fasting has been observed also in the rat. The mobilization of thymus nitrogen produced in the intact male rat by a 96-hour fast was inhibited completely by castration performed 21 days previously, Thus, a loss of 0.61 ± 0.26 mgm.[1] thymus nitrogen per 100 gm. body weight ($p = 0.02$) occurred in the former group. In contrast, no significant change in thymus nitrogen occurred during fasting in the castrate group ($+0.38 \pm 0.6$ mgm.[1] per 100 gm. body weight). In the same experiments, mesenteric nodes from the intact group exhibited no change in nitrogen as a consequence of fasting, whereas those from the castrate animals showed a statistically significant increase in nitrogen concentration ($+0.27 \pm 0.06\%$;[1] $p < 0.01$). Castration appears to exert no influence on the rate of mobilization of nitrogen from spleen.

TABLE 5. THE INFLUENCE OF CASTRATION ON ADRENAL AND PITUITARY WEIGHTS OF FED AND FASTED RATS*

Group	No. of animals	Body weight		Paired adrenals		Pituitary	
		Initial†	Final‡	Wet wt., mgm.	Wet wt., gm./100 gm. B.W.‡	Wet wt., mgm.	Wet wt., gm./100 gm. B.W.‡
Intact Males							
Fed	6	—	329 ± 5	42.5 ± 1.4	12.9 ± 0.5	9.4 ± 0.1	2.84 ± 0.06
Fasted 96 hours	6	319 ± 5	253 ± 10	42.8 ± 2.7	17.2 ± 1.6	8.4 ± 0.3	3.34 ± 0.13
Castrate Males							
Fed	6	—	301 ± 10	56.6 ± 3.4	18.8 ± 0.8	12.7 ± 0.6	4.24 ± 0.23
Fasted 96 hours	6	283 ± 14	214 ± 13	50.1 ± 1.1	23.9 ± 1.6	10.4 ± 0.3	4.90 ± 0.25

* Figures shown are means and their standard errors.
† Body weight at start of 96 hour fast.
‡ Body weight at autopsy.

4. *The influence of castration on pituitary and adrenal weights of fed and fasted rats.*

The use of rats in certain of the inanition experiments made it possible to examine with somewhat greater precision than is possible in mice, the influence of castration on pituitary and adrenal weights of fed and fasting animals. These data are summaried in Table 5. The data obtained confirm the effectiveness of inanition as a stesss in augmenting pituitary-adrenal cortical activity (White and Dougherty, 1947), at least as reflected in the increase in size of the adrenal and the pituitary glands in rats fasted 96 hours, as compared to fed controls. The increase in adrenal weight, resulting from inanition, is not prevented by castration; however, further pituitary enlargement beyond that resulting from castration alone, does

[1] Mean difference between fed and fasted groups, and standard error of the difference.

not occur in the gonadectomized rat fasted for 96 hours. It is to be noted further, in comparison of fed, intact, with fed, castrate rats, that castration in itself produces marked enlargement of the adrenals (cf., Hall and Korenchevsky, 1938; Smith, 1946), and the pituitary (cf., Smith, Severinghaus and Leonard, 1939).

DISCUSSION

Two experimental variables, namely, castration and inanition, have been introduced into the present study. Each of these is associated with profound alterations in metabolism and endocrine function. In turn, each variable exerts an influence upon the effects of the other.

The intact fasting animals utilized in these experiments exhibited the striking sex difference in lipid metabolism to which attention has been directed by previous workers. The net loss of 23% of liver lipid observed in intact fasting male mice is in striking contrast to the accumulation of lipid in that structure in the female. The latter animals exhibited a 74% increase in liver lipid. This is probably best explained on the basis of the augmented rate of lipid mobilization from the depots to the liver in female, as compared to the rate in male, animals during fasting. A sex difference in the processing of lipids by the liver has been revealed by previous investigators (cf., Deuel and Hallman, 1941). Fasting produces a more striking degree of liver lipid increase, ketonemia and ketonuria in the female than in the male. In the present study, the marked rise in liver lipid in the female during fasting was completely abolished by castration. Indeed, fasting female mice showed a net depletion of liver lipid (−43%), and, together with the castrate fasting male, demonstrated that following gonadectomy the loss of fat from the liver during fasting was poorly compensated for by mobilization from the depots. Acceleration of mobilization of fat from the liver by estrogen addition to a diet otherwise low in lipotropic factors has been noted in the rat (György, Rose, and Shipley, 1949). Testosterone under the same conditions was ineffective. This lipotropic effect of estrogen would not be in disagreement with the results of the present study if one interpreted the net accumulation of liver lipid in the fasting intact female as an indication of the accelerated rate of total lipid metabolism. However, it is difficult to reconcile these results with the work of Nyda, di Majo and Lewis (1948), who observed a relative and absolute increase in total body fat of ovariectomized rats treated chronically with estradiol benzoate.

In contrast to the lipid data, the rate of protein loss from the liver, as a consequence of fasting, is unaffected by castration. This finding would appear to constitute evidence that the rate of liver protein mobilization during fasting is as independent of gonadal hormonal influence as it is of

the level of circulating adrenal cortical hormones (cf., White and Dougherty, 1947; Szego and White, 1949). This observation is of interest particularly in connection with the male animals, in which there would be reason to predict that the protein anabolic influence of the androgens might be reflected in a retardation of the rate of loss of nitrogen from the liver during fasting, similar to the manner in which exogenous growth hormone exerts a demonstrable nitrogen-sparing effect in the liver in total inanition (Szego and White, 1949).

The effect of castration on the rate of water loss from hepatic tissue as a consequence of fasting is striking. There was a consistently greater degree of water depletion in the livers of fasted castrate, as compared to fasted intact mice of both sexes. It has been noted in a previous section (cf., Results, 2) that the exaggerated rate of loss of liver water during fasting in castrate animals may be related to the significant retention of this constituent in the gonadectomized controls, as compared with the fed intact animals. The retention of water in the livers of fed castrates in parallel with active lipid deposition is a striking deviation from the usual inverse relationships of these constituents in hepatic tissue during lipid infiltration under other circumstances (cf., Deuel, Hallman and Murray, 1937). Thus, liver lipid accumulation during fasting in the intact mouse, as well as during treatment of intact or adrenalectomized fasting mice with highly purified growth hormone (Szego and White, 1949), is accompanied by a significant decrease in liver water content.

As a consequence of the augmented rate of water and lipid depletion of the liver of the castrate fasted mouse, liver mass decreased more rapidly in this group as compared with the controls, notwithstanding the similar rates of protein mobilization. Fasting thus appears to expose a metabolic defect not otherwise observed in castrate animals.

The data which have been obtained raise certain interesting questions regarding possible quantitative relationships among the various trophic hormones of the adenohypophysis, particularly under circumstances which elicit an over-production of one of these humoral agents. It appears established that castration is followed by augmented production of pituitary gonadotrophins, as reflected in histologic increase in pituitary basophiles, and in an augmented gonadotrophic potency of the gland (Smith, Severinghaus and Leonard, 1939), as well as in an increased excretion of gonadotrophic hormones in the urine. Experimental evidence indicates that the increased gonadotrophin secretion following castration is predominantly the follicle-stimulating hormone (Evans and Simpson, 1950). However, it would appear that there are too few data in the literature to permit the suggestion that castration, with its consequent augmented pituitary secretion of gonadotrophins, is accompanied by either an increased or decreased

production of adrenocorticotrophic hormone. Nevertheless, the results of the present study demonstrate that lymphoid tissue involution, induced as a consequence of starvation, and dependent on pituitary-adrenal cortical secretion (White and Dougherty, 1947), is significantly inhibited in fasting, castrate mice and rats. This would indicate either a diminished secretion of ACTH in the castrate animal subjected to starvation, or a decreased sensitivity of the lymphoid tissues to the dissolution effects (Dougherty and White, 1945) of the adrenal cortical steroids. The data of Evans *et al.* (1938) indicate that lymphoid tissue involution occurs as readily in castrate female rats injected with ACTH as in unoperated controls, suggesting that lymphoid tissue "sensitivity" to adrenal cortical steroids is not reduced by castration. However, Zeckwer (1945), using a severe stress, i.e., formalin injections in castrate rats, observed no retardation of lymphoid tissue involution as a result of gonadectomy. Her data suggest an unimpaired production of ACTH in castrate rats.

The increased gonadotrophin production following castration (surgical or physiological) appears to be followed by a definite suppression of thyrotrophin secretion. Anderson and Kennedy (1933) observed a distinct atrophy of the thyroid in rats three weeks after castration. Loss of gonadal function is also accompanied by a decrease in total activity. This effect may be the primary factor in the increased liver lipids seen in the castrate, fed animals of both sexes in the present study. This increased tendency for castrates to deposit lipid is generally recognized.

While the possible effects of castration, and its consequent increased pituitary production of gonadotrophins, on pituitary ACTH secretion may not be established with certainty, the role of inanition in suppressing gonadotrophin activity of the adenohypophysis appears acceptable. Gonadotrophin assays were significantly lowered in the pituitaries of male rats as a result of chronic inanition (Mason and Wolfe, 1930). The so-called "pseudohypophysectomy" following partial or total inanition has been described by numerous investigators in a variety of species (Moore and Samuels, 1931; Pomerantz and Mulinos, 1939; Sunderman, 1947). It is not yet clear whether this is a consequence of an imbalanced overproduction of pituitary ACTH, as a consequence of the non-specific stress of caloric restriction, or due to an actual deficiency in adequate protein building units for construction of the protein hormones of the adenohypophysis. Since inanition does augment pituitary-adrenal cortical activity (Tepperman, Engel and Long, 1943; Goldzieher, 1946; White and Dougherty, 1947), the data might suggest that the pseudohypophysectomy of inanition is due to the shift of the pituitary to the elaboration of ACTH at the expense of other hormones, e.g., gonadotrophins. A similar suggestion was made by Boutwell, Brush and Rusch (1948) who found that mice restricted in calories were in anestrus, but responded normally to physio-

logical amounts of estradiol in confirmation of similar studies by Huseby *et al.* (1945). However, these animals showed evidence of increased activity of the adrenal cortex. D'Angelo *et al.* (1949) have also obtained data which led them to conclude that starvation in rats and guinea pigs stimulates ACTH production at the expense of gonadotrophins and thyrotrophin.

The suggestion of an imablance in pituitary hormone production caused by the increased release of ACTH during inanition has been supported by recent work of Edelmann (1948) and of Mellgren (1948). The former believes that the gonadal atrophy seen after whole body radiation may be due to a reverse "crowding out" of gonadotrophin production and release by a heavy demand for ACTH. Mellgren also suggested that pituitary basophiles may make ACTH preferentially at the expense of both gonadotrophic and thyrotrophic hormone production.

Selye (1948) has also suggested that overproduction of ACTH may be at the expense of a decreased elaboration of gonadotrophins, luteotrophin, growth hormone, etc., thus leading to an inhibition of somatic growth, involution of the gonads, etc. There can result, therefore, a shift in hypophyseal hormone production under a variety of experimental and physiological circumstances.

SUMMARY

Bilateral gonadectomy was performed in male and female mice of the CBA strain (Strong). Three to four weeks following operation, one-half of the operated animals were sacrificed for analysis of tissue composition, while the other half were subjected to a 48 hour fast, with access to water, and then sacrificed for analysis.

The fed animals, which had been previously gonadectomized, had significantly larger livers than appropriate, intact controls. The composition of the increase was primarily water and lipid. The increase in lipid was disproportionately larger than the increase in either liver weight or water content. These increases occurred at the expense of protein. Due to the increase in liver mass, the diminished nitrogen concentration was not reflected in a significant decline in total liver nitrogen as related to body weight. Castration did not affect the relative mass or composition of lymphoid tissue.

Castration inhibited markedly the mobilization of lipid to the liver, observed in intact, fasted controls. Since the lipid increases in fasting female mice were more significant than in male animals, the effect of castration was more striking in the female. Indeed, the female mouse, previously castrated and then fasted, showed a striking net depletion of liver lipid. Losses of liver nitrogen, during fasting, were unaffected by castration in either sex.

Previous castration of mice or rats also inhibited significantly the pro-

found fasting involution of lymphoid tissue which occurs in unoperated animals. The composition of the absolute decrement in lymphoid tissue weight of castrate animals during fasting was not different from that of unoperated controls. The data are discussed on the basis of the concept of an imbalance which may result in adenohypophyseal secretion as a result of castration (overproduction of gonadotrophins), and in fasting (overproduction of adrenocorticotrophin).

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THE EFFECT OF ACUTE AND CHRONIC DESOXYCORTICOSTERONE ACETATE PRETREATMENT ON THE PITUITARY-ADRENAL RESPONSE TO STRESS[1]

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IN RECENT years considerable interest has been evinced in the factors which control the pituitary-adrenal response to stress. The intact rat responds to such stresses as anoxia (Sacerdote, 1938), benzene (Poumeau-Delille, 1941), ether (Bowman and Muntwyler, 1937) and a host of others too numerous to cite, by a prompt reduction in the quantity of adrenal ascorbic acid. The dominant role of the adenohypophysis in this response is indicated by the fact that its ablation renders the adrenal ascorbic acid unresponsive to stress (Sayers, Sayers, Liang and Long, 1945), although not to the injection of adrenocorticotropic hormone (Sayers and Sayers, 1948).

The finding that the depletion of adrenal ascorbic acid which normally ensues in response to different types of stress may be prevented or inhibited by pretreatment of the animal with cortical extracts and certain of the cortical steroids, in proportion to the dosage employed, has led to the concept of a "peripheral-humoral" control of pituitary-adrenal activity. This postulates that ACTH is discharged by the hypophysis in response to the declining level of circulating cortical hormones, which follows stress as a result of increased tissue utilization or destruction of these steroids (Sayers and Sayers, 1947).

This concept has been challenged by Moya and Selye (1948) who suggest that under conditions of relatively severe stress the response of the pituitary-adrenal axis is conditioned by factors which the theory of peripheral-humoral control fails to explain. Their conclusions, based upon the failure of desoxycorticosterone acetate (DCA) in anaesthetic doses to inhibit the adrenal ascorbic acid depletion following exposure to cold, have been criticized on the grounds that the ascorbic acid drop which they reported was surprisingly small even in the controls, and that the dosage of DCA employed was high enough to be considered toxic and may have been responsible for the discharge of pituitary ACTH (Sayers, 1950).

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[1] This study was aided by a grant from Hoffmann-La Roche Incorporated.

In the present experiments we have reinvestigated this problem, using the more severe stress of scalding. The first experiment was designed to determine if anaesthetic doses of DCA are in themselves stressful and whether or not such doses inhibit the adrenal response to stress. The second experiment was devised in order to determine whether or not chronic treatment with DCA, at a dosage sufficient to cause adrenal involution, would impair the ability of the pituitary-adrenal axis to respond to stress.

EXPERIMENTAL

1. The effect of acute DCA pretreatment on the pituitary-adrenal response to stress.

Thirty-one male albino rats of the Holtzman strain, weighing between 80–100 gm. were divided into two series of 15 and 16 animals respectively. All were fasted for 24 hours, at the end of which time the animals of the first series each received 24 mg. of DCA,* dissolved in 2 cc. of warm corn oil, intraperitoneally; while those of the second series served as controls. Forty-five minutes later, at which time the DCA-treated animals were in a state of deep narcosis, half of them were immersed, except for the head, for 5 secs. in a water bath maintained at 70° C. Similarly half of the animals of the second series were immersed, while under nembutal anaesthesia, for the same length of time.

One hour after stress had been imposed, the scalded rats, together with their controls, were killed by decapitation. At autopsy the adrenals from each animal were carefully dissected and individually weighed to the nearest 0.1 mg. on a torsion balance and immersed in a 6% solution of trichloroacetic acid. Each adrenal gland was analyzed for ascorbic acid content according to the method of Roe and Kuether (1943).

2. The effect of chronic DCA pretreatment on the pituitary-adrenal response to stress.

Thirty-one male albino rats of the Holtzman strain weighing 85–120 gm. were divided into two series. Each of the animals of one series received two 25 mg. pellets of DCA subcutaneously, while those of the other served as controls. The animals were allowed to consume tap water and Purina Laboratory Chow *ad lib.* for a period of 21 days. By this time it was obvious that the animals treated with DCA had developed polydipsia and polyuria.

On the twenty-second day, without a period of fasting, half of the animals in each series were subjected to stress in precisely the same fashion as in the previous experiment. One hour later all of the animals were killed by decapitation. Again the adrenals were weighed, immersed in 6% trichloroacetic acid and duplicate determinations of ascorbic acid in the adrenals of each animal were made.

RESULTS

Table 1 summarizes the pertinent data, together with standard errors, of the acute experiment. It will be observed that there was no significant difference in the body or adrenal weight of the four groups. The adrenal ascorbic acid levels in the stressed group not receiving hormone pretreatment showed a 38% drop from control values. Similarly the stressed

* We are indebted to the Schering Corporation, Bloomfield, New Jersey for the desoxycorticosterone acetate used in these studies.

TABLE 1. THE EFFECT OF ACUTE ADMINISTRATION OF DCA ON ADRENAL RESPONSE TO STRESS

Treatment	Autopsy data			Adrenal ascorbic acid		
	No. of rats	Body wt. gm. ±S.E.	Adrenal wt. mg. ±S.E.	No. of determinations	mg./100 gm. ±S.E.	% decrease
Untreated	8	94±1	20.0±0.7	16	386±11.5	
Scalded 5 sec.* @70° C., 1 hr. before autopsy	8	92±2	19.3±0.7	16	241±10.7 S	38
DCA: 24 mg. in oil, i.p.	8	90±3	20.4±0.7	16	397±25.0	
DCA: 24 mg. in oil, i.p. plus scalding 5 sec. @70° C., 1 hr. before autopsy	7	89±2	20.5±0.5	14	214±14.9 S	46

* Nembutal Anaesthesia.
S = Values statistically different from controls.

animals receiving an anaesthetic dose of DCA showed a drop of 46% from the value observed in hormone treated controls. That the anaesthetic dose of DCA was not "toxic," and that the manipulations of the rat incident to the intraperitoneal injection did not affect the adrenal ascorbic acid values, is indicated by the fact that the values of uninjected controls averaged 386 mg.% while those of the hormone injected group averaged 397 mg. %. Caution was observed at the time of injections to avoid excitement and pain usually associated with such manipulations, which may explain why the adrenal ascorbic acid levels of the hormone injected rats agreed so closely with those of controls. This does not support the contention that anaesthetic doses of DCA may in themselves elicit ACTH discharge from the anterior pituitary.

Table 2 summarizes the data, together with the standard errors, obtained in the chronic experiment. It is apparent that the dosage of DCA employed, while not affecting the body weight of the rats, did produce a significant amount of adrenal atrophy. Again, as in the previous experiment, DCA *per se* had no effect on the concentration of adrenal ascorbic acid, despite

TABLE 2. THE EFFECT OF CHRONIC ADMINISTRATION OF DCA ON ADRENAL RESPONSE TO STRESS

Treatment	Autopsy data				Adrenal ascorbic acid		
	No. of rats	Body wt. gm. ±S.E.		Adrenal wt. mg. ±S.E.	No. of determinations	mg./100 gm. ±S.E.	% decrease
		Initial	Final				
Untreated	8	100±4	190±6	27.2±1.1	16	444± 8.7	
Scalded 5 sec.* @70° C., 1 hr. before autopsy	8	101±3	203±6	26.6±1.2	16	322±7.0 S	27
DCA: 2–25 mg.* pellets implanted 3 weeks previously	7	100±4	188±8	22.4 ±1.1S	10	446±14.7	
DCA: 2–25 mg.* pellets implanted 3 weeks previously plus scalding 5 sec. @ 70° C., 1 hr. before autopsy	8	101±5	190±5	21.8 ±0.8S	15	283±12.0 S	37

* Nembutal Anaesthesia.
S = Values significantly different from controls.

the adrenal atrophy induced by this chronic form of treatment. Similarly it failed to inhibit in any way the decline of adrenal ascorbic acid evoked by stress. The adrenal ascorbic acid values in the rats stressed, but not receiving DCA, showed a decline of 27% from control values: while the adrenal ascorbic acid in the DCA-implanted group subjected to stress was 37% below that of the non-stressed, but hormone treated controls.

DISCUSSION

Our data are incompatible with the view that "peripheral-humoral" control is the sole factor involved in the response of the pituitary-adrenal axis to stress, insofar as the action of DCA may be considered representative of cortical steroid activity.

Cheng and Sayers (1949) have advanced the hypothesis that the increased insulin sensitivity manifested by demedullated rats subjected to prolonged DCA overdosage is a consequence of cortical insufficiency resulting from continuous pituitary inhibition. This argument has also been advanced to account for the hypoglycemia to which fasting DCA-treated rats are particularly susceptible (Selye and Dosne, 1942). Insofar as the ascorbic acid drop consequent to stress is a reliable index of actual hormone secretion by the adrenal cortex, and this theory is not entirely unassailable (Vogt, 1948; Long, 1947), our results do not support the thesis that either acute or chronic overdosage with large quantities of DCA in any way impairs the ability of the cortex to respond to severe stress. Chronic overdosage with DCA however caused a considerable amount of cortical atrophy. Whereas the data indicate that a comparable amount of ascorbic acid, and presumably cortical hormone, was discharged per unit of tissue there was also less tissue. Consequently less total ascorbic acid was present and the hormone secretion may have been reduced in the DCA-treated animals. This argument obviously does not apply in the acute experiment.

While these experiments were in progress a report appeared suggesting that two mechanisms may be involved in pituitary ACTH control (McDermott, Fry, Brobeck and Long, 1950). These may be independent or sequential depending upon the conditions of stress employed, and have been termed the autonomic and the metabolic phase respectively. The former depends upon the reflex secretion of epinephrine whereas the latter it is postulated, although without direct proof, is related to the rate of utilization or destruction of circulating adrenal cortical hormones. The nature and timing of our experiments were such that we may have been dealing primarily with the autonomic phase of ACTH secretion which may not be affected by the level of circulating cortical hormones.

In both the acute and chronic experiments as may be seen in Tables 1

and 2, the DCA-pretreated groups responded to stress with a greater drop in adrenal ascorbic acid from control values than did the animals subjected to stress alone. In view of the fact that in neither case is the difference of unquestionable statistical significance, it would be presumptuous to attempt to draw definite conclusions from the results, but in view of the fact that in both cases these tended in the same direction and were mutually supportive we feel that some comment is warranted. Obviously this cannot be ascribed to a "toxic" action of DCA *perse*, since the adrenal ascorbic acid levels in the non-stressed animals treated with the hormone did not differ from those of controls. It may be that while DCA alone is not stressful, pretreatment with the hormone may sensitize the animal to stress.

SUMMARY

Desoxycorticosterone acetate, whether administered acutely in anaesthetic doses, or chronically in a dosage sufficient to cause polydipsia, polyuria and adrenal cortical atrophy, does not affect adrenal ascorbic acid concentration. Such dosages do not in themselves constitute a stress.

The hormone, when given either acutely or chronically, fails to impair the ability of the pituitary-adrenal axis to respond to stress with the usual drop in adrenal ascorbic acid values.

Experiments with DCA lend no support to the "peripheral-humoral" concept of pituitary-adrenal control as it applies to severe stress.

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GROWTH-INHIBITING EFFECT OF CORTISONE ACETATE ON THE CHICK EMBRYO

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IN THE course of a study of the effects of various agents on the development of the chick embryo (Karnofsky, Patterson and Ridgway, 1949, 1950, Karnofsky, Ridgway and Patterson, 1950), it was found that 11-dehydro-17-hydroxy-corticosterone acetate (cortisone acetate) caused a striking inhibition of embryonic growth and development (Karnofsky, Stock and Rhoads, 1950). This observation was anticipated by Landauer (1947), who found that an extract of the adrenal cortex, injected into the yolk sac of the 0, 1 or 5 day chick embryo markedly inhibited its subsequent growth. This report presents further studies on the growth-inhibiting effects of cortisone, when injected into the yolk sac or onto the chorioallantoic membrane of the chick embryo at various stages of development.

MATERIALS AND METHODS

Fertile white Leghorn eggs were obtained from a commercial source. They were incubated at 38° C. and 75 per cent relative humidity. The eggs were kept in one position, and moved once daily when candled for viability. Cortisone (11-dehydro-17-hydroxy-corticosterone acetate) was available in an aqueous suspension.[1] Yolk sac injections were made through a hole drilled in the blunt end of the egg, and then the opening was sealed with melted paraffin. The chorioallantoic membrane was injected through a small window cut in the shell directly over the membrane and, after the injection, the opening was sealed with Scotch tape. Embryos were injected at 0, 2, 4, 6, 8, 10, 12, 13 and 15 days of development. The surviving embryos were sacrificed at various times after injection, but principally at 18 days of age, and then weighed and

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[1] Cortisone was made available by Merck and Company in an aqueous suspension containing 25 mg./cc, and appropriate dilutions of this suspension were made before use. Cortisone was preserved in 1.5 per cent benzyl alcohol. In experiments with benzyl alcohol it was found that about 10 mg. benzyl alcohol was the minimum dose necessary to produce detectable injury in the 4 day embryo, whereas the amount received by the embryo injected with 4 mg. cortisone (the largest dose used) was about 2 mg. of benzyl alcohol.

examined grossly. Embryos show a definite but irregular spontaneous mortality during incubation, and this may be increased by the trauma of the injection procedure or by the introduction of infection. This variable spontaneous mortality must be considered in analyzing the relationship of the dose of cortisone, the route of injection and the age of the embryo at time of injection to the per cent of embryo deaths due directly to the action of cortisone. Our data on the comparative activity of cortisone under different conditions, therefore, were derived chiefly from the weight and appearance of embryos surviving until 18 days; when embryos receiving the higher doses of cortisone died before

FIG. 1. The appearance of a normal and cortisone-treated embryo sacrificed at 18 days of incubation. The cortisone-treated embryo was injected into the yolk sac with 4 mg. of cortisone acetate at 4 days. It is small, feather formation is scant, and the eyes are prominent.

18 days and showed clear-cut evidence of a "cortisone effect" (Fig. 1), it was presumed that death was due to the action of cortisone, and these data were included in our analysis. In some experiments embryos were sacrificed at various ages, and body weight was used as the sole index of cortisone activity.

RESULTS

Effect of cortisone on the chick embryo. The dose of cortisone used in the chick embryo ranged from 0.10 to 4.0 mg./egg, and the amount of cortisone

necessary to inhibit embryonic growth varied with the age of the embryo at the time of injection, and with the route of injection. Effective doses of cortisone given before the 10th to 12th days of incubation produced characteristic changes in the embryo, which could be recognized easily in chick embryos surviving to the 14th day or beyond.

The effects resulting from large doses of cortisone early in development were first seen at about the 8th to 10th day of incubation when, on candling the egg, a marked restriction was noted in the extent of the chorioallantoic membrane and yolk sac. Many of the embryos, showing severe inhibition of membrane formation and vascularization died before the 18th day but, even in those embryos surviving to 18 days, often about one-half or less of the surface of the egg was covered by the chorioallantoic membrane.

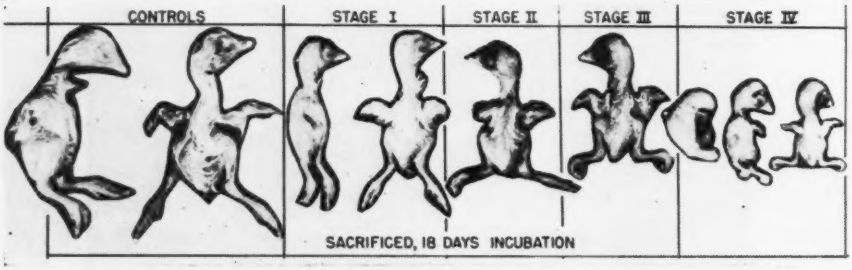

FIG. 2. The range of growth-inhibition and embryo abnormalities produced by various doses of cortisone acetate. Cortisone acetate was injected into the yolk sac of the 4 day embryo, and the embryos were sacrificed 14 days later. *Stage I*. Slight stunting, with suggestion of scanty feather formation on head. *Stage II*. Slight to moderate stunting, moderate baldness of the head, and sometimes with clubbed feathers on the head and body. *Stage III*. Moderate stunting, with almost baldness of the head, and scanty feather formation with clubbed feathers on the body. *Stage IV*. Typical cortisone syndrome, described in text.

In the embryos surviving to 14 days of age and beyond, marked inhibition of embryonic growth was apparent. The embryos were sacrificed routinely at 18 days of incubation, and the following description applies to embryos at this stage of development. On opening the egg, an excess of free yolk and a large amount of albumen were present. The yolk was incompletely surrounded by membrane, and the albumen was free. Occasionally, the incomplete yolk sac was small and the yolk within it was inspissated, whereas the unincorporated yolk was fluid.

The embryos surviving to 18 days of age after the maximum tolerated dose of cortisone showed marked stunting in development and the characteristic appearance shown in Fig. 1. The embryo was curled up, and the amnion drawn tightly around it. The beak was pressed tightly against the

left wing. The embryos were pale and, occasionally, mildly edematous. Embryos dying before 18 days often showed a gross milky edema. The eyes were disproportionately large, and sometimes protruded from the orbit. Some of the embryos had exteriorized viscera. Feather formation was markedly deficient, and almost completely absent in some cases.

Smaller doses of cortisone produced lesser degrees of stunting and abnormalities in development. Figure 2 shows the degrees of inhibition in growth and development of 18 day embryos produced by a range of doses of cortisone given by the yolk sac route at 4 days. These effects were arbitrarily divided into four stages, and the criteria for each stage are given in Figure 2. Although the "stage" of developmental inhibition

TABLE 1. CORRELATION OF THE "STAGE" AND WEIGHT OF CHICK EMBRYOS TREATED WITH ADRENOCORTICAL STEROIDS

"Stage"	No. embryos	Average weight, 18 days, gm.	Standard deviation
0	50	16.4	±2.6
I	23	13.4	±2.4
II	32	11.5	±1.6
III	22	9.0	±1.5
IV	40	6.5	±2.0

is graded separately from the weight of the embryo (measure of growth inhibition), the two effects are, obviously, directly related. The average weights of the embryos at the different "stages" shown in Figure 2 are given in Table 1.

Procedure for estimating cortisone activity in the chick embryo. The striking effect of cortisone (Fig. 1), and the range of the effects of intermediate doses on embryonic weight and development (Fig. 2) provide a qualitative and a roughly quantitative method for estimating its hormonal activity in the chick embryo. A semi-quantitative method is thus available for assaying the activity of cortisone injected by different routes at various stages of embryonic development. There are considerable variations in the weights of embryos from commercially obtained eggs incubated under identical conditions, and occasionally small or abnormal embryos may be found in untreated eggs at 18 days. Furthermore, many agents will interfere with embryonic growth, so that growth inhibition alone can not be taken as evidence of a specific effect of cortisone. Only Stage IV, shown in Figures 1 and 2, is regarded as characteristic of cortisone activity and this effect must be present in a given experiment before the consistent occurrence of the intermediate degrees of growth inhibition (Stages I and II, particularly) produced by lower doses can be attributed to its action.

The following procedure for the assay of cortisone activity was used:

1. The dosage range, in most cases, was based on double increments; i.e., 0.5, 1.0 and 2.0 mg./egg. Considering the wide variations in embryonic effects obtained at a given dose of cortisone, these intervals were regarded as adequate.

2. The eggs were candled daily, and the dead embryos examined. If the dead embryos had survived long enough to show a Stage IV effect, these "stage" data were used in the analysis. Embryos surviving to 18 days

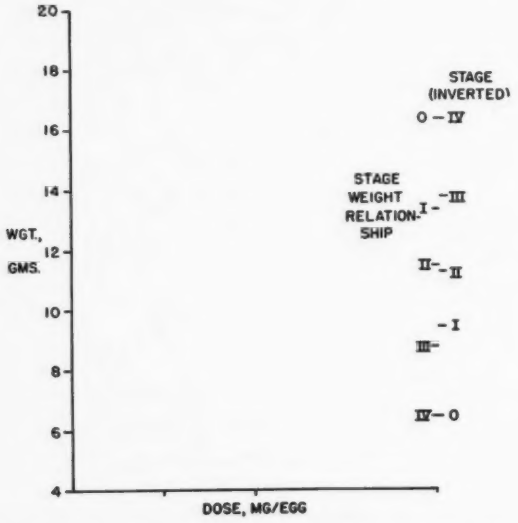

FIG. 3. Chart used for plotting data on quantitative effects of cortisone acetate in the chick embryo.

were sacrificed, weighed and graded according to the criteria presented in Figure 2.

3. The body weight and stage of growth inhibition for each embryo at each dose level were plotted separately on the graph form shown in Figure 3. Embryo weight is on the left side ordinate and the "embryo stage" on the right. The dosage, as mg./egg, is plotted on the abscissa. The "stage" scale is plotted in relation to the average embryo weight as shown in Table 1. The "stage" scale, however, was then inverted, so that the weight and stage data on a normal embryo and on an embryo showing the "cortisone syndrome" were at opposite ends of the chart. This method allowed an easier inspection of the data and, by plotting the average weights and "stages" at each dosage level, a crossing of these lines occurred when cortisone activity was present. This intersection is arbitrarily taken to

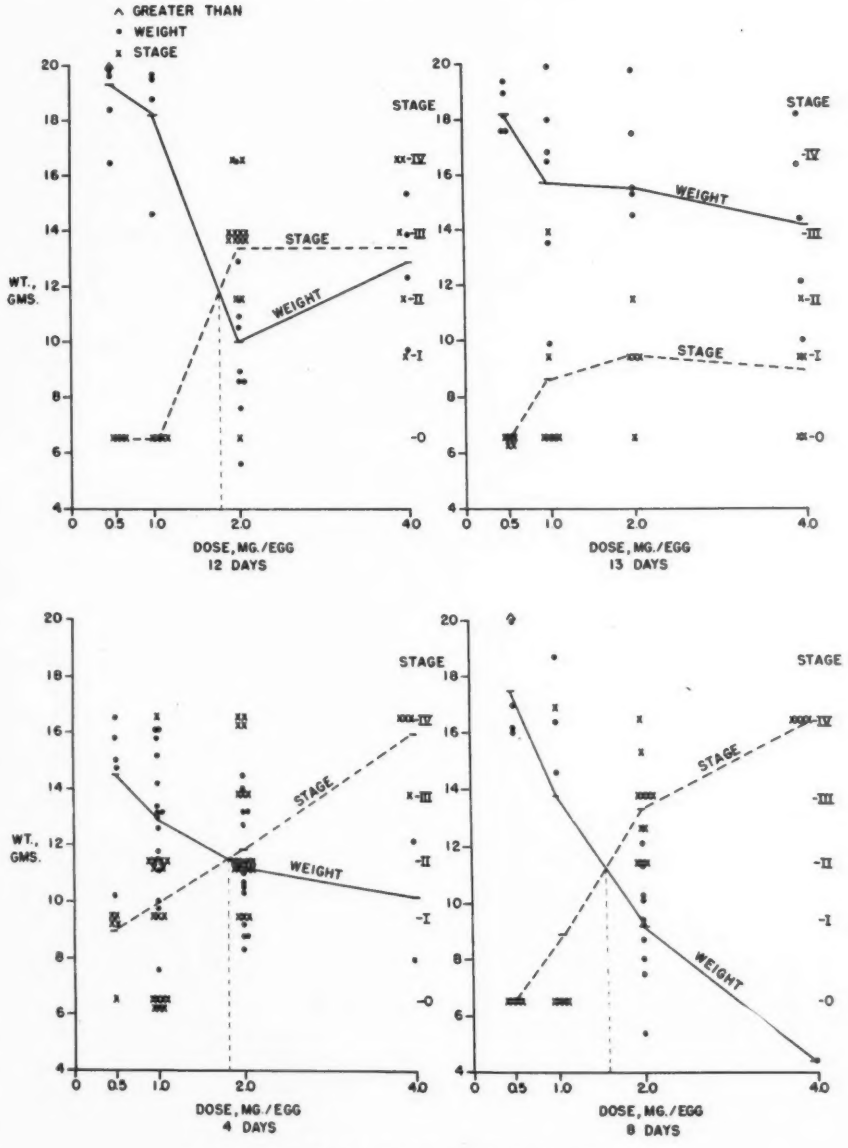

FIG. 4. The effects of cortisone by yolk sac injection in the 4, 8, 12 and 13 day chick embryo.

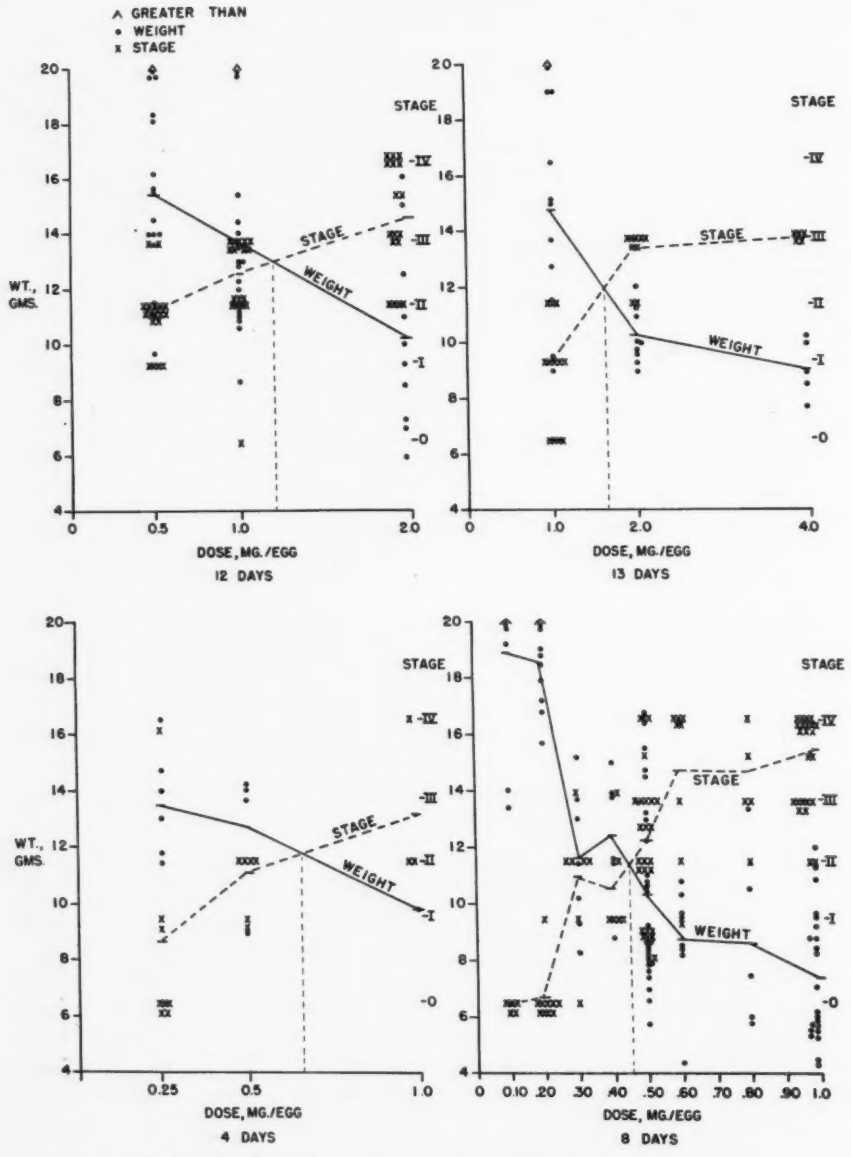

FIG. 5. The effects of cortisone injected onto the chorioallantoic membrane of the 4, 8, 12 and 13 day chick embryo.

represent the *minimum effective dose* which will produce the cortisone effect. In Figures 4 and 5 the intersection occurred at an average weight of 11.8 gm. (11.3–13.0 gm. range) and average "stage" of 2.1 (1.9–2.6). It must be emphasized, however, that the *minimum effective dose* is valid only if, at a higher dose, cortisone produces the Stage IV effect.

Activity of cortisone when injected into the yolk sac at various stages of development. The activity of various doses of cortisone injected into the yolk sac of embryos 4, 8, 12 and 13 days of age, in terms of embryo mortality and the degree of growth inhibition in embryos surviving to 18 days, is given in Table 2 and Figure 4. In the embryos injected at 4, 8, 12

TABLE 2. MORTALITY IN CHICK EMBRYOS INJECTED WITH CORTISONE
(Mortality rate is based on the per cent deaths occurring up to and including the 18th day of incubation)

Dose cortisone, mg./egg	Age of embryo at injection, days			
	4	8	12	13
	Number eggs injected/number dying (% mortality)			
		Yolk sac injection		
0.5	6/ 1 (17)	6/ 2 (33)	6/ 2 (33)	6/1 (17)
1.0	22/ 6 (27)	6/ 2 (33)	6/ 2 (33)	6/0 (0)
2.0	29/15 (52)	19/ 9 (47)	15/ 6 (40)	6/1 (17)
4.0	10/ 8 (80)	4/ 3 (75)	6/ 2 (33)	6/1 (17)
		Chorioallantoic membrane injection		
0.10		5/ 0 (0)		
0.20		11/ 2 (18)		
0.25	12/ 6 (50)			
0.30		8/ 1 (13)		
0.40		6/ 0 (0)	6/ 0 (0)	
0.50	12/ 8 (67)	32/ 7 (22)	17/ 1 (6)	12/2 (17)
0.60		8/ 8 (0)		
0.80		6/ 1 (17)	6/ 0 (0)	
1.00	12/11 (92)	37/15 (40)	18/ 1 (5)	12/0 (0)
2.00		25/20 (80)	20/10 (50)	12/3 (25)
4.00		4/ 4 (100)		6/1 (17)

and 13 days of age, the LD_{50} *dose* of cortisone was approximately 2.0, 2.0, 4.0 and 4.0 mg. and the *minimum effective dose* was 1.8, 1.5, 1.8 and 4.0 mg./egg, respectively. The effects of a single dose of cortisone given between the 4th and 12th day were fairly constant; however, cortisone appeared to be slightly more active when injected at 8 days, and there was a definite decrease in activity when it was given at 13 days. This latter observation may explain the discrepancy in Figure 4, where the 12 day embryo treated with 4 mg. of cortisone showed a lesser effect than the embryos receiving 2 mg.; the 4 mg. dose was given to a different group of embryos, and it is possible that they were some hours older at the time of injection and, therefore, less susceptible to the action of cortisone. The LD_{50} and the minimal effective doses of cortisone injected at 4 and 8

days were almost parallel, and only a few embryos showing the Stage IV effect survived to 18 days. Many of the deaths following cortisone occurred within the first 3 days after injection, or when the embryos were 17 to 18 days old. The embryos injected at 4 days showed a peak of mortality at 7 to 10 days, which may be of significance.

Activity of cortisone when injected onto the chorioallantoic membrane at various stages of development. Various doses of cortisone were applied directly to the chorioallantoic membrane of the 4, 8, 12 and 13 day old embryo, and the surviving embryos were sacrifiecd at 18 days of age. The mortality data, and the effects on the embryos surviving to 18 days, are given in Table 2 and Figure 5, respectively. In embryos injected at 4, 8, 12 and 13 days of age, the LD_{50} *dose* of cortisone was approximately 0.25, 1.2, 2.0 and 4.0 mg. and the *minimum effective dose* was 0.65, 0.45 1.20 and 1.60 mg./egg, respectively. Some differences in the activity of cortisone are suggested by the results from chorioallantoic membrane as compared with those from yolk sac injection.

1. Cortisone was unusually toxic when applied to the chorioallantoic membrane of the 4 day embryo. While chorioallantoic membrane injection at this age produces an immediate high mortality (11/70 (16%) of the control injected embryos and 19/108 (18%) of the embryos injected with 1 mg. of cortisone died within 24 hours), during the next 2 days 5 (9%) of the 54 remaining controls and 16 (22%) of the remaining cortisone-treated embryos died. It is probable that the higher mortality in the latter group is attributable to the effect of cortisone. The few embryos receiving 1 mg./egg at 4 days on the chorioallantoic membrane and surviving to 18 days were stunted and eviscerated, but they did not show the complete cortisone effect seen in the embryos injected at 8 days, or by the yolk sac route at 4 days, since considerable feather formation was present.

2. When injected at 4 and 8 days of age, cortisone was about 3 times more active by the chorioallantoic membrane as compared to the yolk sac route. The most marked effect of cortisone can be produced by chorioallantoic membrane injection at 8 and 12 days with doses which permit the embryo to survive to 18 days, and the LD_{50} *dose* is about twice the *minimum effective dose*. Thus by chorioallantoic membrane injection at 8 days, the Stage IV "cortisone effect" (Fig. 2) can be produced regularly in embryos surviving to 18 days without the mortality seen following yolk sac injection.

3. At the 13th day of incubation, cortisone showed a reduced activity, although it was still more active by the chorioallantoic route. As with yolk sac injection, the activity of cortisone was greatest when given on the 8th day.

Effects of single vs. fractionated doses of cortisone in the chick embryo.

Since a single dose of cortisone was used in the previous experiments, it was of interest to determine if fractionating the dose of cortisone over several days modified its effectiveness. These experiments were carried out by chorioallantoic inoculation. In one experiment, cortisone was given at a single dose of 1 mg./egg to 4 and 8 day embryos and, in a 3rd group, 0.2 mg./egg was given daily for 5 days starting in the 4 day embryo. The results indicated that the fractionated dose of cortisone had a greater activity than the single total dose, and the embryos surviving beyond 14 days resembled those embryos injected with a single dose at 4 days, in that they had more feathers than those given a single dose at 8 days.

In another experiment, single doses of 0.5 and 1.0 mg. of cortisone were given to 8 and 12 day embryos, and a 3rd and 4th group received 0.1 mg.

TABLE 3. COMPARISON OF THE ACTIVITY OF SINGLE AND FRACTIONATED DOSES OF CORTISONE ACETATE IN THE CHICK EMBRYO
(Embryos sacrificed at 18th day of incubation)

Single dose				Fractionated dose, daily ×5			
Dose, cortisone, mg./egg	No. embryos	Aver. wgt. gm. (range)	Aver. "stage" (range)	Daily dose cortisone, mg./egg	No. embryos	Aver. wgt. gm. (range)	Aver. "stage" (range)
Injected 8 days				*Injected 8 to 12 days*			
0.5	8	11.9 (5.8–16.8)	2.1 (1–4)	0.1×5	12	8.6 (4.5–10.8)	3.0 (2–4)
1.0	11	7.8 (4.3–10.9)	3.5 (3–4)	0.2×5	9	5.7 (4.1– 7.3)	4.0
Injected 12 days							
0.5	6	15.3 (11.4–18.3)	1.7 (1–2)				
1.0	6	11.8 (8.7–14.4)	3.0				

and 0.2 mg. daily, beginning at 8 days for a period of 5 days. The results are shown in Table 3. It is apparent that the fractionated doses of cortisone were more effective than the same total dose given at 8 days, and the effects of cortisone on the embryo corresponded closely to those seen when a single injection was given at 8 days.

Cortisone, thus, appears to be more active when given in fractionated daily doses than when given as a single total dose, and the morphological effects obtained with fractionated doses over a 5 day period resembled those to be expected if the single total dose were given at the time of the initial fractionated dose.

The initiation of the cortisone effect. The maximum effects of cortisone seen in embryos surviving beyond 14 days were fairly uniform, irrespective of whether the embryos had been injected at 4, 8, or 12 days of age. This suggested that cortisone was not acting immediately on injection, and exerting a more profound effect on the younger embryo, but that it exerted its action at a particular and, presumably, advanced stage of development. The following experiments were carried out in order to localize

more accurately this developmental period. Most of the studies were performed by yolk sac injection, since the chorioallantoic membrane is not developed until the 4th day.

The three experiments reported below indicate that cortisone exerts its effect on embryonic development on the 8–10th day of development; the onset of the effect apparently varying to some extent with the dose employed.

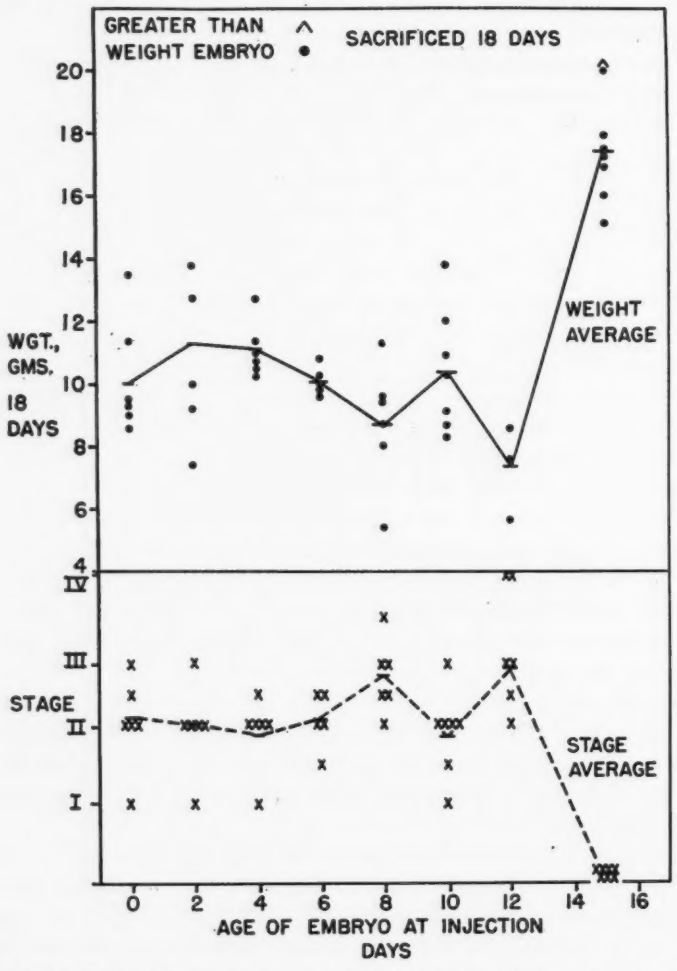

FIG. 6. The effects of 2 mg. cortisone injected into the yolk sac of the 0, 2, 4, 6, 8, 10 and 12 day chick embryo; all sacrificed at 18 days. (15 day embryo received 4 mg./egg.)

Cortisone was injected into the yolk sac of 0, 2, 4, 6, 8, 10, 12 and 15 day old embryos, and the surviving embryos were sacrificed at 18 days. The dose in all but the last group was 2 mg./egg; the 15 day embryos received 4 mg./egg. The results are presented in Figure 6. The embryos treated at 0 to 12 days showed approximately the same degree of weight and growth inhibition (Fig. 2) when examined at 18 days, and the embryos injected at 15 days with 4 mg. of cortisone per egg were not appreciably affected. In this experiment, a given dose of cortisone produced a fairly uniform effect on late embryonic development when given at any time up to the 12th day of development.

In another experiment, 2 mg. of cortisone was injected into the yolk sac of 2 day old embryos, and then groups of 5 to 6 embryos were sacrificed when they reached 4, 6, 8, 10, 12, 14, 16 and 18 days of age. The weights of these embryos were compared with those of controls injected at 2 days with 0.1 cc. of a 1.5 per cent solution of benzyl alcohol in saline. The average weights, with their standard deviations, of the treated and control embryos, are plotted in Figure 7. Until the 10th day of development, the weights of the two groups are parallel; in fact, the cortisone-treated embryos are heavier than the controls on the 6th and 8th days. At the 12th day and beyond, the cortisone-treated embryos showed a progressive decrease in the rate of growth, and at 18 days the control embryos averaged 21.5 gm. and the cortisone-treated embryos 13.8. gm These data indicate that the inhibition of growth produced by the yolk sac injection of cortisone is first manifested during the 9th to 11th days.

In the third experiment, to further clarify the time of onset of the cortisone effect in the chick embryo, 4 mg./egg of cortisone was injected into the yolk sac of 6, 8, 10 and 12 day embryos, and 5 or 6 embryos in each group were sacrificed and weighed at one to 2 day intervals after injection, up to 14 days. Appropriate control embryos were sacrificed at the same time. The entire experiment was not carried out on a single batch of eggs, and control embryos were sacrificed for the several separate runs. Consequently, instead of the actual embryo weights, the combined data are presented in Figure 8 as the percent deviation of the cortisone-treated embryos from their proper controls. It is clear that embryos injected at the 6th and 8th days showed only slight inhibition of growth on the 10th day but, by the 12th day, all embryos, including those injected on the 10th day, showed a marked decrease in the rate of growth. These data again implicate the 10th day as the period when cortisone, injected into the yolk sac, produces its first evidence of growth inhibition.

Observations by chorioallantoic membrane injection. The time of onset of the cortisone effect, when cortisone was applied directly to the chorioallantoic membrane, was determined in the following experiment. One mg.

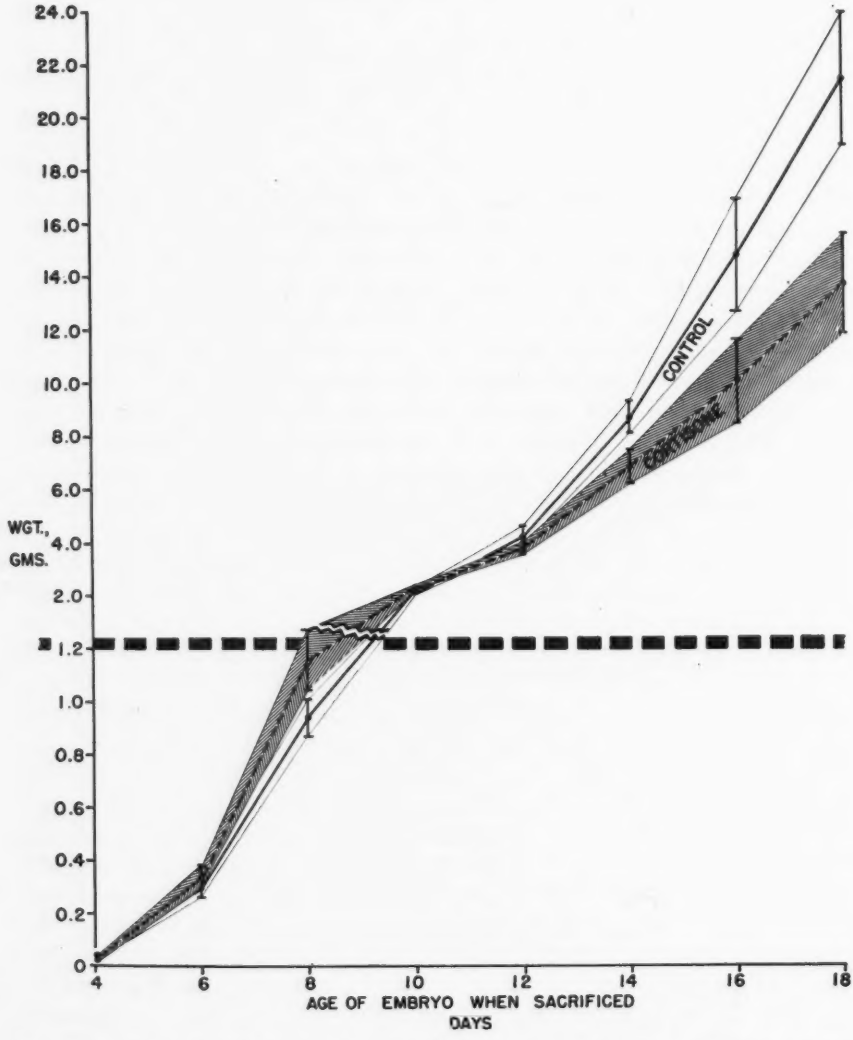

FIG. 7. The effects of 2 mg. of cortisone injected into the yolk sac at 2 days, on the rate of growth of the chick embryo. (Average weight and standard deviation of control and cortisone-treated embryos.)

cortisone per egg was applied to the chorioallantoic membrane of the 4 day embryo, and 5 to 7 eggs were sacrificed at 5, 6, 7, 8, 10 and 14 days of age and the embryos weighed. The data are presented graphically in Figure 9. Cortisone, applied to the chorioallantoic membrane, had an immediate growth-inhibiting effect, and this effect was particularly conspicuous in marked but transient inhibition in the extension of the chorioallantoic membrane. This immediate effect of cortisone, when added to the chorioallantois at 4 days, was confirmed in several other experiments. After the immediate growth-inhibiting effects, the treated embryos began

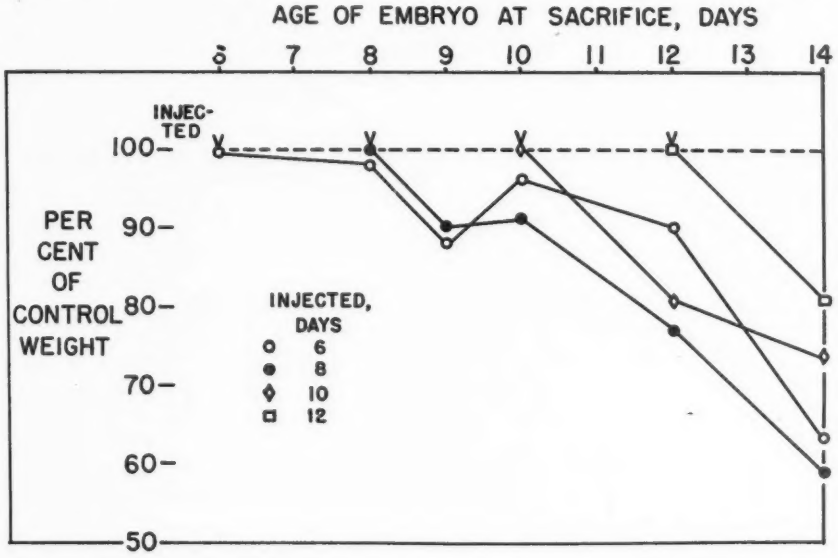

FIG. 8. The per cent decrease in growth rate of embryos injected into the yolk sac with 4 mg. of cortisone as compared to controls. (Sacrificed at one to 2 day intervals.)

to increase in weight at an approximately normal rate, although at the 10th day they still lagged behind the controls, weighing 1.87 gm. per embryo as compared to 2.28 gm. for the controls; thus, weighing 18 per cent less than the controls. After the 10th day, the "cortisone effect" was evident, and the cortisone-treated embryos, sacrificed on the 14th day, averaged 5.8 gm. in weight as compared to 10.3 gm. for the controls; the treated embryos, thus, weighing 44 per cent less than the controls. This experiment indicated that cortisone applied to the chorioallantoic membrane had an immediate but transient effect on the growth of the chorioallantoic membrane and embryo, but the characteristic growth-inhibiting effect of cortisone was not evident until after the 10th day.

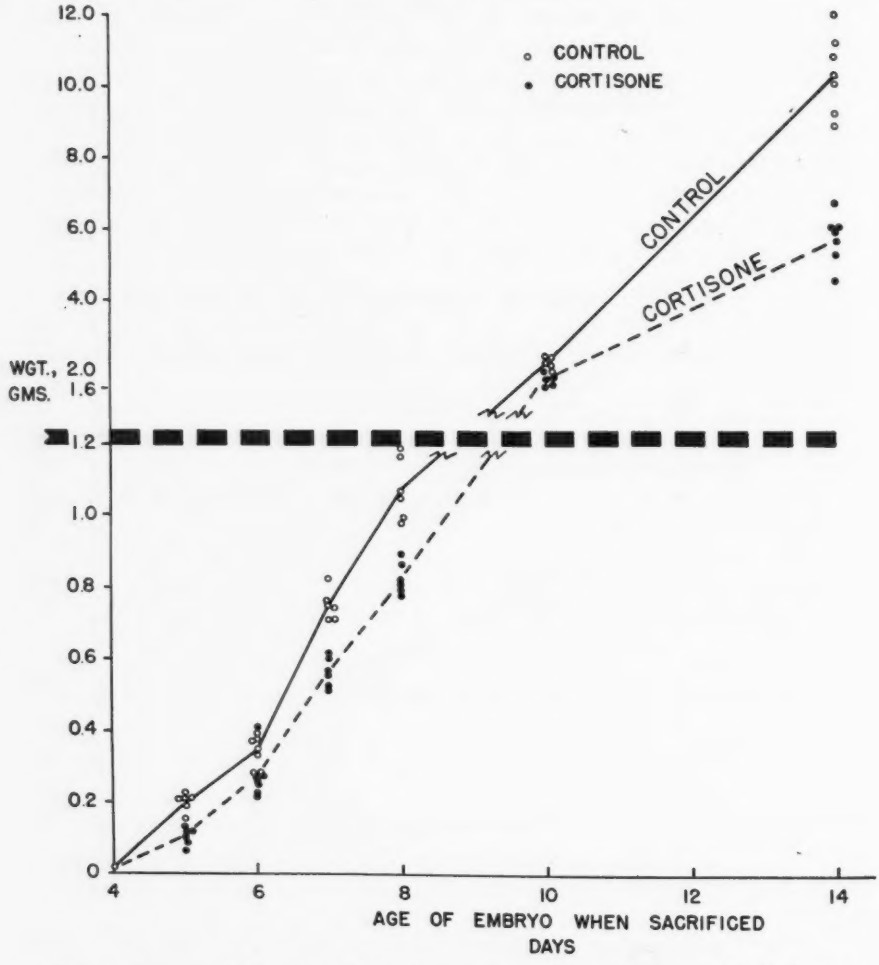

FIG. 9. The growth-inhibiting effect of 1 mg. of cortisone applied to the chorioallantoic membrane of the 4 day old embryo. The weight of each control and cortisone-treated embryo sacrificed at 5, 6, 7, 8, 10 and 14 days is plotted separately.

DISCUSSION

The growth inhibiting effect of cortisone was described in rats by Kendall and Wells (1940), and the subject has been reviewed recently by Ingle (1950). Cortisone also inhibits the growth of the chick embryo, producing characteristic effects which are distinct morphologically and toxicologically from those caused by other chemicals such as the folic

acid antagonists, 2,6-diaminopurine (Karnofsky, Patterson and Ridgway, 1949) and anti-vitamin B_6 (Karnofsky, Stock, Ridgway and Patterson, 1950), which became less active with the increasing age of the embryo, thallium (Karnofsky, Ridgway and Patterson, 1950) and lead (Ridgway Patterson and Karnofsky, 1950) which exert their effects on specific tissues of the embryo, and nitrogen mustard (Karnofsky, Burchenal, Ormsbee, Cornman and Rhoads, 1947), which causes immediate injury that is either rapidly fatal, or from which, in most cases, complete recovery ensues.

The mechanisms of action of cortisone on the chick embryo are unquestionably complicated, and two types of effects seem probable: a local inhibitory effect, as evidenced by the action of cortisone applied locally to

TABLE 4. SUMMARY OF THE TOXICITY AND GROWTH-INHIBITING EFFECTS OF CORTISONE WHEN INJECTED BY YOLK SAC AND CHORIOALLANTOIC MEMBRANE ROUTES INTO THE 4, 8, 12 AND 13 DAY EMBRYO (SEE TABLE 2 AND FIGS. 4 AND 5)

Age at inject., days	*Yolk sac route*				*Chorioallantoic membrane route*			
	Approx. LD_{50}	Minimum effect, dose	Aver. wgt.,* Embryo, gm.	Aver.* "stage"	Approx. LD_{50}	Minimum effect, dose	Aver. wgt.,* embryo, gm.	Aver.* "stage"
	mg./egg				mg./egg			
4	2.0	1.8	11.5	2.0	0.25	0.65	11.8	2.4
8	2.0	1.5	11.3	1.9	1.2	0.45	11.4	1.9
12	4.0	1.8	11.7	2.1	2.0	1.2	13.0	2.6
13	4.0	4.0	—	—	4.0	1.6	- 11.9	2.1

* The average weight and "stage" are the effects on the embryo estimated to be produced by the *minimum effective dose* of cortisone (Figs. 4 and 5). (Embryos were sacrificed at 18 days of incubation.)

the chorioallantoic membrane of the 4 day embryo in temporarily inhibiting the growth of the membrane and the embryo and, secondly, the generalized and prolonged inhibition of embryonic development which appears to be initiated at the 8th to 10th day of development. The toxicities and minimum effective doses of cortisone producing growth inhibition of the embryo when given by the chorioallantoic and yolk sac routes at 4, 8, 12 and 13 days of age are summarized in Table 4. The LD_{50} of cortisone by yolk sac injection is in the same range as the *minimum effective doses* for the 4 and 8 day embryos. The *minimum effective doses* of cortisone at 4 and 8 days by the yolk sac route are about 3 times higher than by the chorioallantoic route, but at 12 days the activity of cortisone by these two routes of injection approach each other. In the 4 day embryo cortisone is very toxic when applied to the chorioallantoic membrane, and this toxicity is particularly conspicuous when the LD_{50} dose is compared to the dose producing the "cortisone effect" in the embryo surviving to 18

days; when given at the 8th to 12th days, however, the embryos survive until the 18th day on doses of cortisone by the chorioallantoic route which produce a maximum inhibition in embryonic development. These data suggest that cortisone may be exerting a local action on the chorioallantoic membrane of the 4 day embryo, and this local effect is responsible for the lethal and transient growth-inhibiting action of cortisone. The larger doses of cortisone necessary to inhibit embryonic development, when given by the yolk sac as compared to chorioallantoic membrane route, may be due to the dilution of cortisone in the yolk, or to its slow or delayed absorption from the yolk sac.

The action of cortisone which is first evident at the 8th to 10th day of development, is seen as a marked and persistent inhibition in growth rate of the chick embryo and the extraembryonic membranes. The restriction in membrane growth, which is possibly secondary to the interference with embryonic growth, undoubtedly aggravates the degree of embryonic inhibition by interfering with the absorption of yolk and albumen. Cortisone is effective when given as a single dose, although divided doses are somewhat more active (Table 3). This observation suggests that a single dose of cortisone, injected into the closed system of the egg, is exerting a continuous action on the embryo. The marked increase in the resistance of the embryo to cortisone on the 13th day of development and thereafter suggests that the embryo is now able to metabolize cortisone, or that embryonic systems, previously very sensitive to cortisone, have now matured and become more resistant.

The evidence that the embryo is not susceptible to the action of cortisone until the 8th to 10th day of development may provide a clue to its mechanism of action. The 8th to 10th day period in development, however, is a complex one and involves remarkable changes in embryonic metabolism, which are summarized by Needham (1942). At this period liver activity is initiated; the embryonic liver deposits glycogen (Dalton, 1937) and cholesterol (Dalton, 1937a), there is a sharp rise in liver peptidase (Dumm and Levy, 1949), and nitrogenous wastes, which were first excreted as ammonia and then urea, now appear as uric acid (Needham, 1942). The embryo, which used glucose as its source of energy early in development, now begins to burn fat, although for a short period there is an increase in protein catabolism. A general awakening of the endocrine glands occurs during this period. The pituitary (Rahn, 1939), thyroid (Woodside, 1937), adrenal cortex (Dawson, 1949), and islands of Langerhans (Needham, 1942) show histological evidence of activity and estrogenic hormone may be recovered from the embryo at 10 days (Needham, 1942). Hypophysectomy of the 2 to 3 day embryo does not immediately affect growth and development (Fugo, 1940), but the embryos show a progressive decrease

in growth after the 10th day, feather formation continues in a relatively normal manner, and some of the embryos survive beyond the normal hatching period. Similarly, depressing the activity of the thyroid gland with thiourea (Adams and Bull, 1949), decreases embryonic growth although feather formation is not seriously affected, and the embryo often survives beyond the normal hatching period. Hyperthyroidism causes a decrease in the growth of the embryo (Willier, 1924). Insulin, injected into the yolk sac at 2, 4 and 5 days of age, produces striking developmental abnormalities (Landauer, 1947a), and a prolonged fall in blood sugar which does not return to normal levels until the 12th to 14th days, when insulin secretion presumably begins (Zwilling, 1948). Adreno-cortical extract injected into the yolk sac at 5 days causes a rise in blood sugar reaching its peak at 10 to 12 days, but when insulin is given simultaneously a delayed but intensified and more prolonged hypoglycemia occurs than that following insulin alone. While the onset of endocrine activity in the embryo appears to coincide with the initiation of the cortisone effect, it is important to emphasize that the changes in the embryo produced by excesses of thyroid or sex hormones (Snedecor, 1949), insulin and epinephrine (Landauer, 1947), or deficiencies of the pituitary and thyroid functions do not resemble the embryonic abnormality obtained with cortisone.

In searching for an explanation for the onset of the "cortisone effect" of the embryo on the 8th day, it seems unlikely that cortisone acts primarily by inhibiting or modifying the function of the endocrine glands or that, at this period, the embryonic tissues abruptly become susceptible to the action of cortisone. More promising suggestions are that cortisone may either specifically inhibit the formation of a new function or tissue, essential for growth, normally appearing at the 8th to 10th days, or interfere with the utilization of a new substrate or substrates which are provided for cell growth as a result of the shift in embryonic metabolism.

It is further noted that the effects of cortisone described in the embryo may be the end-result of a chain of events several steps removed from the primary action of cortisone. For example, if the cortisone-treated embryo is unable to utilize its food supply, embryo starvation becomes an important factor in producing the final picture of the cortisone effect. Cortisone may produce embryo starvation by directly interfering in the absorption of yolk, or directly inhibiting the growth of the extra-embryonic membranes, or by inhibiting the growth of the embryo itself, so that it only absorbs the food it is able to use.

The marked inhibition of *feather development* produced by cortisone seems to be of particular interest. Dermal papillae are the organizers of feather follicles (Lillie and Wang, 1944), and they begin to appear in the 8 day embryo. Subsequently, the ectodermal component of the feather is

stimulated, and Goff (1949), has recently described this process in detail. Cortisone almost completely inhibits the formation of feathers, although a few follicles, presumably present before the cortisone effect begins, continue to grow. Following the injection of cortisone at 8 days or earlier, the embryos examined at 18 days of age may show gross evidence of interruption in feather formation at about the 10 day stage, although body weight may have increased to the 13 or 14 day level. These results suggest that cortisone is inhibiting selectively the formation of the dermal papillae, but it does not prevent the further growth of feather follicles that have already formed.

The postulated inhibition of feather follicle induction by cortisone is consistent with the observation of Ragan, Howes, Plotz, Meyer and Blunt (1949), that the mesodermal components of healing wounds in rabbits are inhibited by cortisone. This effect of cortisone on feather formation may be secondary to a more specific effect on the embryo, or it may be due to the local action of cortisone on a tissue highly sensitive to its action. The mechanism of the cortisone effect on feather formation further emphasizes the complex problems presented in attempting to analyze the action of this hormone in the chick embryo.

Practically, however, the 8 day embryo may serve as a qualitative and semi-quantitative method for detecting the "cortisone-like activity" of other steroid hormones. The suggested assay procedure in the egg consists in injecting a single dose of the test substance onto the chorioallantoic membrane of the 8 day embryo, since cortisone exhibits its greatest activity when given at this time. The term "cortisone-like activity" applies to the effect produced by cortisone in the chick embryo; but this effect is not necessarily related to steroid activity as measured by other assay methods. It is already known that some steroids have different activity in the chick embryo as compared to their activity by other assay techniques (Karnofsky, Bartle, Ridgway and Patterson, 1950).

SUMMARY AND CONCLUSIONS

1. Single injections of cortisone acetate, 0.2 to 4.0 mg./egg, depending on the route and time of injection during embryonic development, will interfere with the growth and development of the chick embryo. The effects of cortisone on the embryo, even when injected before incubation, appear to be initiated on the 8th to 10th days, and the embryo beyond this period shows progressive inhibition in growth and development.

2. When applied to the chorioallantoic membrane of the 4 day old embryo, cortisone seems to have a transient inhibitory effect on the membrane and the growth of the chick embryo, which lasts 24 to 48 hours. The embryo then proceeds to recover and grows at about the same rate as the control,

until the 10th day after which growth inhibition of the embryo recurs.

3. At the maximum dose of cortisone permitting the embryo to survive to 18 days, a specific and striking syndrome is produced. The embryo is small, extra-embryonic membrane formation is inhibited, and the absorption of yolk and albumen is diminished. The embryo shows almost complete absence of feather formation, while the eyes, brain and heart seem to be disproportionately larger. Smaller doses of cortisone produce less severe effects on growth and feather formation. These embryos may survive until 20 to 21 days, the time of hatching, but none have survived beyond this period.

4. Cortisone is approximately 3 times more effective in the 4 and 8 day embryo when injected on the chorioallantoic membrane as compared to yolk sac injection, and it shows its greatest effect on embryonic development when given at 8 days. The minimum effective dose at 8 days by the chorioallantoic route is estimated to be 0.45 mg./egg.

5. The possible reasons for the initiation of the cortisone effect at the 8th to 10th days, and the mechanism of the cortisone effect are discussed.

6. The chick embryo may be useful as a preliminary, qualitative, and semi-quantitative method for assaying other substances, for effects on the chick embryo similar to cortisone.

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INHIBITION OF DCA INDUCED HYPERTENSION AND HYALINOSIS BY A DRUG ACTING ON MEMBRANE PERMEABILITY

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THE sodium content of the diet has been conclusively shown to be an essential factor in the pathogenesis of DCA (Desoxy-Corticosterone-Acetate)—induced hypertension and associated morphological alterations (Selye, 1942; Selye and Pentz, 1943; Selye, Mintberg and Rowley, 1945), as such changes are prevented on a salt free diet (Selye, Stone, Timiras and Schaffenburg, 1949). Furthermore high doses of NaCl are conducive in some species to hypertension and hyalinosis even in the complete absence of mineralo-corticoids (Selye, and Stone, 1943). The Na ion would thus seem to be the main responsible agent, salt retaining steroids merely facilitating its action. This facilitating role of DCA and related steroids could be ascribed to altered membrane permeability (Fine and Fischmann, 1950) leading to an increased content of intra-cellular sodium at the level of the proximal convoluted tubules. A so-called antihistaminic compound, (1-dimethyl-amino-2′methyl, 1′ethyl-N-) dibenzo-p-thiazinehydrochloride also known as 3277 R.P. or Phenergan, has been shown to have opposite effects on membrane permeability (Halpern, 1949; Leduc, 1949). It therefore appeared of interest to determine wether its administration could prevent the development of hypertension and associated morphological lessions in DCA treated animalc.

MATERIAL AND METHODS

96 female piebald rats of an average weight of 60g at the onset of the experiment were unilaterally nephrectomized and given 1% NaCl to drink for maximal sensitization. The animals were subdivided into five groups, which were treated as follows: three groups were administered DCA; of these one received it alone while the other two were treated in addition with Phenergan and histamine hydrochloride respectively. Two control groups were administered histamine and Phenergan alone. DCA was given in three successive weekly subcutaneous implantations of one 35 mg. pellet. Phenergan (5 mg. p.d. in aqueous solution) was subcutaneously injected twice daily. Histamine hydro-

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* Research fellow of the French Government's Relations Culturelles.

† This work was done during the tenure of a Research Fellowship of the American Heart Association.

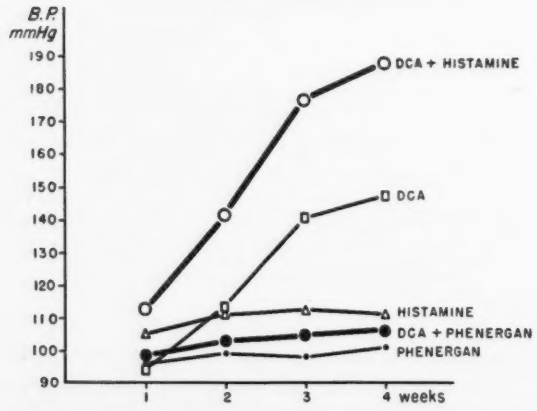

FIG. 1. Effect on the blood pressure of DCA, Phenergan and histamine administered alone or concurrently.

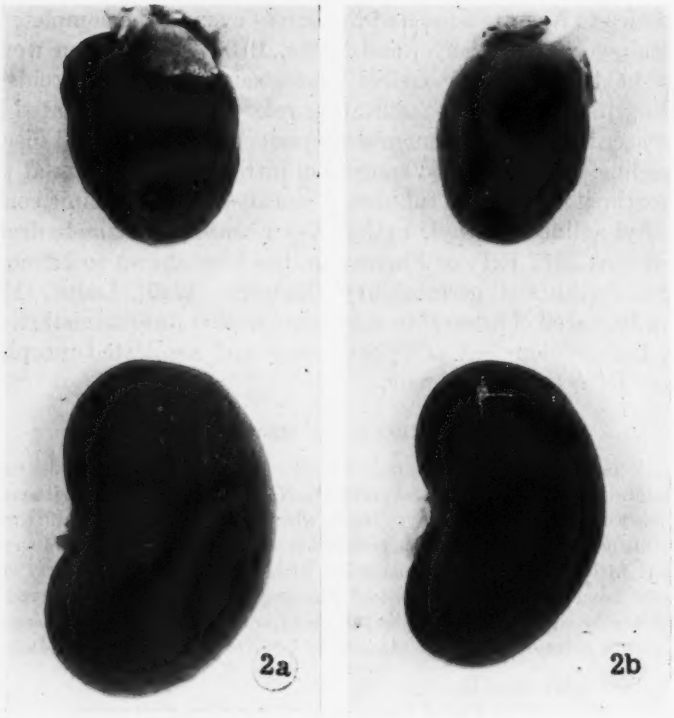

FIG. 2. Hearts and kidneys of animals treated with DCA (2a), and DCA and Phenergan (2b). ×450.

chloride, used as a means of obtaining paired weight controls through its growth inhibiting and stressing effect, was administered at a progressive dosage of 0.6 to 2 mg. p.d. Fluid intake was measured daily, body weight and blood pressure once a week, the latter by the tail method with Sobin's microphonic device. After 30 days the animals were killed and the organs macroscopically examined. In addition the kidneys and hearts were fixed, weighted and stained with H & E.

RESULTS

a) *Blood pressure*

As can be seen from Fig. 1, DCA progressively increased the blood pressure level; neither Phenergan nor histamine alone had any effect, while,

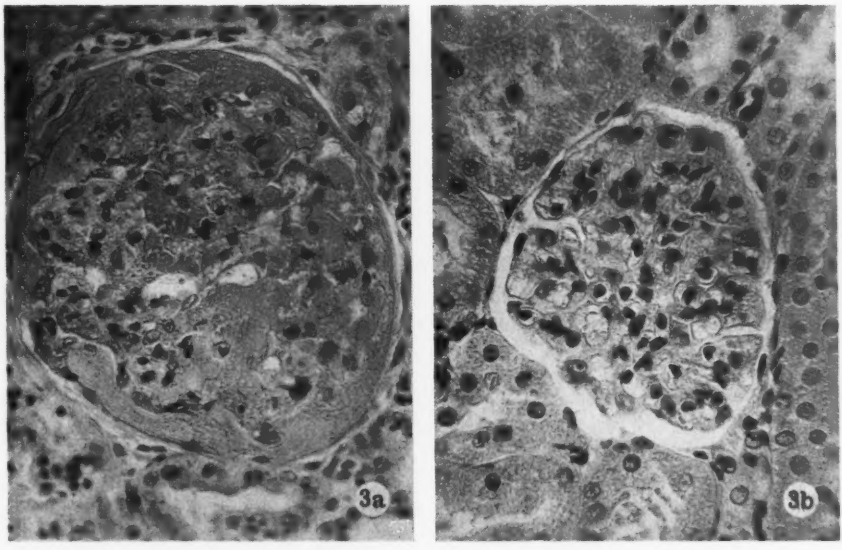

FIG. 3. Glomerular hyalinosis in DCA treated animal (3a). Normal appearance of the kidney in a rat receiving DCA and Phenergan (3b). ×450.

concurrently administered with DCA, Phenergan prevented and histamine markedly increased its hypertensive effect.

b) *Fluid intake*

The water (1% NaCL) intake of the DCA treated animals was markedly reduced by the administration of Phenergan.

c) *Morphology*

Myocardial nodules, periarteritis nodosa as well as macroscopic signs of nephrosclerosis were observed at autopsy in most of the animals treated

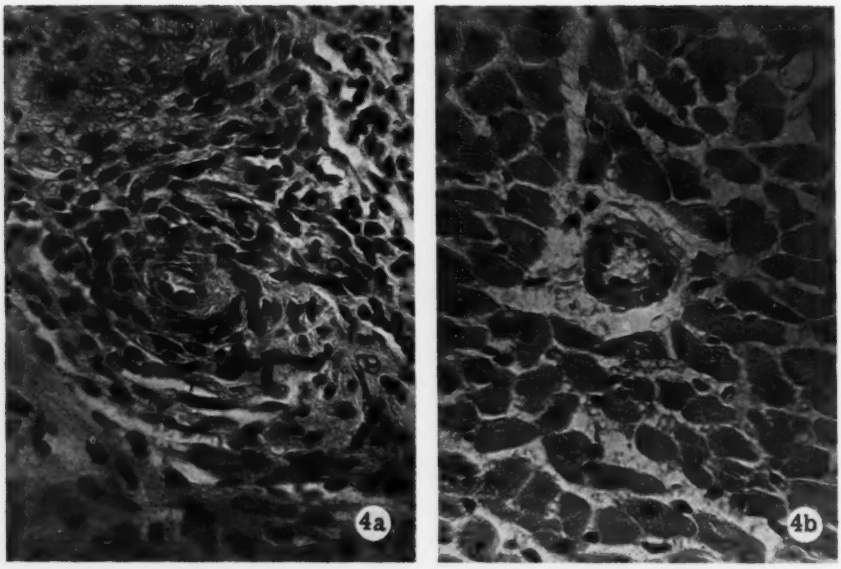

FIG. 4. Cardiac periarteritis nodule in a DCA treated rat (4a) normal arteriole in the heart of an animal treated concurrently with DCA and Phenergan (4b). ×450.

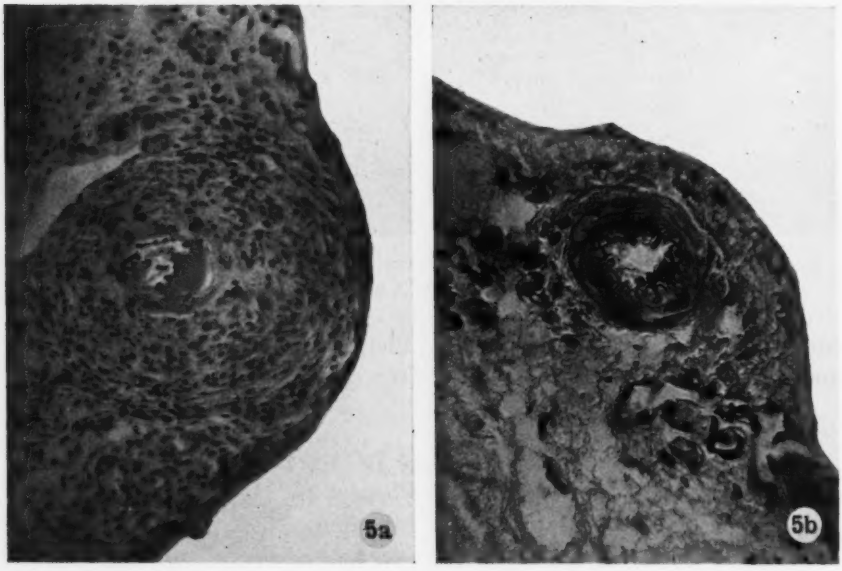

FIG. 5. Periarteritis nodosa in the mesentery of a rat treated with DCA (5a). Normal appearance of a mesenteric vessel in a rat receiving DCA and Phenergan (5b). ×450.

with DCA alone or in association with histamine (Fig. 2). With a single exception (where nephrosclerosis was present) no such changes could be observed in the animals concurrently given Phenergan and DCA. Histological findings confirmed these observations. The DCA treated animals showed in all cases, besides glomerular hyalinosis, an intense tubular enlargement and cellular vacuolization predominent in the proximal segment. Phenergan, with the above mentioned exception, completely prevented the development of both glomerular and tubular lesions (Figs. 3a and 3b). Futhermore Phenergan administration completely inhibited the formation of cardiac or mesenteric nodules of periarteritis (Figs. 4a & 4b, 5a & 5b). Body and organ weights are shown in Table 1.

TABLE 1. EFFECT OF DCA, PHENERGAN AND HISTAMINE ON BODY AND ORGAN WEIGHTS

Treatment	Body weight		Heart, mg./100 g. B.W.	Kidney, mg./100 g. B.W.
	Initial	Final		
DCA[1]	55 ± 1	112 ± 3	540 ± 14	1030 ± 37
Phenergan[2]	57 ± 5	122 ± 15	382 ± 5	632 ± 21
Histamine[3]	53 ± 5	97 ± 4	443 ± 7	827 ± 11
DCA + Phenergan	55 ± 6	100 ± 12	557 ± 21	978 ± 45
DCA + Histamine	53 ± 2	90 ± 1	616 ± 24	1260 ± 35

[1] 3 successive weekly subcutaneous implantations of a 35 mg. pellet.
[2] 5 mg. p.d. in aqueous solution, subcutaneously injected twice daily.
[3] Histamine (HCl) in aqueous solution, subcutaneously injected twice daily at a progressive dosage of 0.6 to 2 mg. p.d.

DISCUSSION AND CONCLUSIONS

The functional as well as the morphological changes induced by DCA overdosage were satisfactorily prevented by the concurrent administration of Phenergan.* This is ascribed chiefly to the antagonistic action of the drugs on membrane permeability and the consequent effects on intra-cellular sodium content. The possibility that Phenergan might have decreased the absorption of DCA pellets should nevertheless be considered. Our result should be related to clinical observations showing the favorable therapeutic effects of antihistaminic drugs in cases of acute glomerular nephritis (Craig, Clark and Chalmers, 1949) and pre-eclamptic hypertension with albuminuria (Hoffman, 1949). Further clinical studies along these lines would be indicated. The use of Phenergan or related drugs in the initial stages of essential hypertension would warrant special interest.

* Before this paper was submitted for publication, a report has appeared (Zacco, M. and Ciasca, G., Rheumatismo 3: 1, 1950) which corroborates our observations regarding the preventive action of antihistaminic drugs on DCA induced morphological alterations in the rat.

SUMMARY

The concurrent administration of Phenergan (3277 R.P.) and DCA in sensitized animals prevented the development of the functional and morphological changes associated with DCA overdosage. A theoretical explanation of these results is suggested.

Acknowledgments

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THE EFFECT OF GROWTH HORMONE ON HEPATIC CATALASE, AND ON CELL VOLUME AND HEMOGLOBIN CONTENT OF BLOOD IN RATS[1]

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RELATIONSHIPS of the growth hormone to metabolic processes are being actively studied. Its glycostatic and ketogenic effects (Wilhelmi, Fishman, and Russell, 1948; Bennett *et al.*, 1948), probable implication in glucose uptake by diaphragm (Park and Krahl, 1949) and anti-insulin action in hypophysectomized dogs (De Bodo *et al.*, 1950) have recently received attention. Since the metabolism of protein is interrelated with that of carbohydrate and fat, clues as to the mechanism by which the growth hormone influences protein synthesis may well develop from studies of the above type, which are concerned with other aspects of metabolism. Consequently, we have felt that our inquiry into effects of growth hormone on tissue enzymes, though begun with a view of elucidating the mechanism by which well-known effects on body weight, skeletal development, and nitrogen storage are produced, might well include assays of enzymes having no obvious relationship to these criteria of growth. Accordingly, tissues from a series of animals used for studies of enzyme systems involved in skeletal development (Mathies and Gaebler, 1949) or in protein metabolism and metabolism of muscle (Gaebler *et al.*, 1949) were also used for investigation of renal and hepatic acid and alkaline phosphatases (Mathies *et al.*, 1949) and for some of the present observations on hepatic catalase. Because the changes in catalase activity were striking, effects of growth hormone on another heme-containing protein functionally unrelated to catalase became of interest. Determinations of cell volume and hemoglobin of the blood were therefore also included.

EXPERIMENTAL

At first, catalase was determined in the whole homogenate according to the Jolles modification of the method of von Euler and Josephson as employed by Miller (1948), except that different dilutions were required to obtain K_0 values in the valid range (Sumner and Somers, 1947). As the liver homogenates employed in the assay were stored, at times, at −18° C. for 1 to 2 weeks before use, control tests were carried out

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which showed that no significant reduction of activity occurred on standing at 0° C. for 22 hours, followed by storage at −18° C. for 46 days. These results were to be expected in view of the great stability of this enzyme when not greatly diluted. Assays were carried out in duplicate with the exception that in group II, Table 2, the average value was obtained from single assays in each animal. As this study progressed, it was noticed that homogenates autolyzed at 0° C. for several hours gave less erratic values than very fresh ones. Presumably, this was due to more complete extraction of the enzyme from cell particulates.

During this investigation Feinstein (1949) published a method for catalase estimation which is considerably better than previous ones. In the last series of animals (Table 3) the analyses were therefore made exactly as described by Feinstein.

Total nitrogen determinations were carried out on the whole homogenate by the micro-Kjeldahl procedure of Hiller, Plazin and Van Slyke (1948). Cell volume and total hemoglobin determinations were carried out on the day of sacrifice, using blood obtained by heart puncture and, as anticoagulant, the concentration of ammonium and potassium oxalates found satisfactory for rat blood by Heller and Paul (1935). The Van Allen hematocrit was used for cell volume determinations, while optical density of hemoglobin solutions was measured spectrophotometrically at 540 mμ.

Diets and feeding techniques are described in preceding studies. Growth hormone preparations were assayed in plateaued female rats by the firm which supplied them. For purposes of comparison, it is of interest that two preparations designated as containing 3502 and 1740 growth units per gram were reassayed in our laboratory, using immature female hypophysectomized rats 26 to 30 days old. The daily doses required to produce, in 10 days, an average gain in weight of 1 gm. per day were 11 gammas and 26 gammas respectively. Thus the potency of the preparation designated as containing 3502 growth units approximated that reported for purified growth hormone (Fishman, Wilhelmi and Russell, 1947).

RESULTS

Five experiments on mature normal white rats, of 2 strains and both sexes, are summarized in Table 1. In only 2 groups (III and V, Table 1) were there significant decreases in hepatic catalase after treatment with growth hormone. However, it should be noted that decreases, whether significant or not, occurred in 4 of the 5 experiments. It appears that in Sprague-Dawley rats treated with growth hormone a small depression of hepatic catalase occurs irrespectively of gain in weight, method of calculation, or technique of feeding. Of particular interest is the occurrence of a significant change in group V, for these male rats showed no significant gain in weight. Possibly, the longer period of treatment is the explanation.

Results obtained in mature intact animals were therefore suggestive, but by no means conclusive. Data from 2 experiments on immature hypophysectomized rats, which are more responsive, are summarized in Table 2. In one of these experiments, the operated animals were fed *ad lib.*, in the other one the treated hypophysectomized animals were pair-fed against the hypophysectomized controls. A group of normal controls of the same age, sex and strain was pair-fed against each group of hypophysectomized

TABLE 1. THE EFFECT OF GROWTH HORMONE ON HEPATIC CATALASE OF PLATEAUED RATS*

Experiment	No. of rats	Average of body wt. at start of injection period in gm.	Average wt. gained during injection period in gm.	Average terminal liver wt. in gm.	Catalase units		
					Per gm. tissue	Per mg. total N	Per 100 gm. body wt.
I. 8 month old Sprague-Dawley females, pair-fed. Given 3 mg. growth hormone (1485 R.U./gm.) per day per rat for 7 days.							
Treated	5	251	13	6.13	580		1360
Controls	5	253	5	6.64	620		1610
P‡					>0.7		>0.4
II. 6 month old "Edwards" females. *Ad lib.* fed. 6 mg. of above growth preparation given daily per rat for 6 days; 12 mg. on the 7th day.							
Treated	5	202	24	8.26	1260		4580
Controls	5	208	1	7.34	1200		4180
P					>0.6		>0.3
III. 8 month old Sprague-Dawley females, pair-fed. Given 6 mg. growth hormone (1740 R.U./gm.) per rat per day for 7 days; 12 mg. on the 8th day.							
Treated	5	256	21	8.44	910	25.3	2770
Controls	5	264	4	7.79	1150	32.3	3340
P					<0.01	<0.01	<0.05
IV. 8 month old Sprague-Dawley females, pair-fed. Given 6 mg. growth hormone (1740 R.U./gm.) per rat per day for 7 days.							
Treated	4	244	12	7.20	980		2780
Controls	5	257	1	7.57	1190		3470
P					<0.2 <0.1		<0.2 >0.1
V. 8–10 month old Sprague-Dawley males. *Ad lib.* fed; 10 mg. growth hormone (1740 R.U./gm.) given per day per rat for 14 days. Fasted 24 hrs. before sacrifice.							
Treated	5	481	16†	10.96	1340	33.4	2930
Controls	5	471	4†	11.13	1800	43.8	4220
P					<0.01		<0.01

* Diet was Rockland rat diet (powdered for the paired-feeding experiments).
† Gain by the 13th day.
‡ Fisher (1948).

rats. That rapid growth occurred can be seen from the listed gains in weight (Table 2). Because catalase assay results were independent of the manner of calculation, the statistical significance of the differences between groups is given only for the data calculated on a unit per gram basis.

The data in Table 2 indicate that hepatic catalase activity is 2 to 3 times as great in the hypophysectomized control groups as in their pair-fed normal controls, and in both experiments the results are highly significant statistically. Treatment of operated groups with the indicated amounts of growth hormone resulted in a reduction of this high value—or maintenance of a low value—for both *ad lib.* and pair-fed groups of treated hypophysectomized rats had hepatic catalase values about 50% below those of hypophysectomized controls. These results are, again, very highly significant statistically. When the treated hypophysectomized rats are compared with the treated normals, or the normal controls, the two groups cannot be statistically differentiated in the first experiment, while in the second one a difference with a low order of significance exists. Thus, in hypophysectomized rats treated with large doses of growth hormone preparations, hepatic

catalase is reduced to, or maintained at, very nearly normal levels. As was the case in experiments presented in Table 1, comparison of normal groups in Table 2 presents a variable result, with a suggestive decrease in the treated group of the first experiment.

Three additional series of rats, 1 month old at operation but otherwise comparable with the groups in Table 2, were examined. Analysis of pooled livers disclosed the usual very high catalase concentration following hypophysectomy in 2 of the series but not in the third. A final experiment was, therefore, carried out in which the more satisfactory catalase estimation of Feinstein (1949) was employed, and in which other factors such as the dosage of growth hormone and the possible effect of inanition in normal animals pair-fed against hypophysectomized ones were also considered. The results are reported in Table 3. It is obvious that hepatic catalase activity in the operated rats is far above that of the normal controls, whether the latter are pair-fed or fed *ad lib.* Furthermore, this high activity was not strikingly reduced by small doses of a fairly pure preparation of growth hormone which produced almost as much gain in weight as occurred in the *ad lib.* fed normal controls. The significance of this will be discussed further. Incidentally, the food intake of controls fed *ad lib.* was nearly double that

TABLE 2. THE EFFECT OF GROWTH HORMONE ON THE HEPATIC CATALASE OF NORMAL AND HYPOPHYSECTOMIZED RATS

Experiment	No. of rats	Average body wt. at start of injection period in gm.	Average weight gained during injection period in gm.	Average terminal liver wt. in gm.	Catalase units*		
					Per gm. tissue	Per mg. total N	Per 100 gm. body wt.
I. Sprague-Dawley female rats, two months old at operation. Diet of Bennett *et al.* (1948) Hypophysectomized rats fed *ad lib.*: normals pair-fed against them (B1 fed against A1; B2 against A2). Plateaued for 14 days after operation and then injected 1 mg. growth preparation (1384 R.U./gm.) daily per rat for 17 days.							
A. Hypophysectomized							
1. Treated	6	154	53	6.80	1520 ± 360	46.5 ± 12.9	5100 ± 1680
2. Controls	5	146	−1	4.65	2760 ± 410	76.3 ± 12.3	8780 ± 1190
B. Normals							
1. Treated	5	175	25	5.45	1090 ± 410	28.9 ± 11.0	3030 ± 1280
2. Controls	5	165	−1	4.27	1390 ± 260	35.9 ± 6.5	3580 ± 460
II. Same as above, except a 24 day injection period was used and all rats were pair-fed against group A2.							
A. Hypophysectomized							
1. Treated	5	176	46	5.39	990 ± 220	27.1 ± 6.2	2390 ± 600
2. Controls	7	173	5	5.80	2010 ± 350	59.2 ± 14.5	6570 ± 1290
B. Normals							
1. Treated	5	189	23	4.86	670 ± 130	17.4 ± 3.2	1540 ± 260
2. Controls	6	191	2	4.62	660 ± 190	18.1 ± 5.5	1590 ± 490

* Given with standard deviation from the mean.

Significance of the differences between the groups: (Fisher, 1948) Expt. I—A1, A2, $P<0.01$; A1, B1, $P<0.2>0.1$; A2, B2, $P<0.01$. Expt. II—A1, A2, $P<0.01$; A1, B1, $P<0.05>0.02$; A2, B2, $P<0.01$. These data are calculated for units per gm. tissue results.

TABLE 3. DATA SHOWING THAT HEPATIC CATALASE IS NOT LOWERED IN NORMAL RATS BY RESTRICTION OF FOOD INTAKE, AND IS NOT REDUCED TO NORMAL IN HYPOPHYSECTOMIZED RATS RECEIVING SMALL DOSES OF GROWTH HORMONE

Group	No. of rats	Average body wt. at start of injection period in gm.	Average weight gained during injection period in gm.	Average daily food intake during injection period in gm.	Average terminal liver weight in gm.	Catalase activity per gm. tissue*
Sprague-Dawley female rats, 2 months old at operation. Diet of Bennett *et al.* (1948). Hypophysectomized rats fed *ad lib.*; B1 normals pair-fed against A1; B2 normals fed *ad lib.* Plateaued 26 days after operation. Group A2 then received intraperitoneally 20 gammas growth preparation (3502 R.U./gm.) daily per rat for 10 days. Rats were 3 months, 1 week old when sacrificed.						
A. Hypophysectomized						
1. Control	5	119	+ 3.4	7.0	4.08	800 ± 124†
2. Treated	5	126	+16.2	7.6	4.93	727 ± 84
B. Normals						
1. Controls	5	139	+ 2.0	7.0	3.27	475 ± 32
2. Controls	5	211	+18.8	14.4	7.91	484 ± 32

* Millequivalents perborate decomposed (Feinstein, 1949).

† Significance of the differences (Fisher, 1948): A1, A2, $P<0.4>0.3$; B1, B2, $P<0.7>0.6$; A1, B1, $P<0.01$; A1, B2, $P<0.01$.

of the other 3 groups, but there is no significant difference between the hepatic catalase of pair-fed and *ad lib.* fed normal groups. The high values in hypophysectomized animals are therefore not an artifact produced by lowering of values in the normal controls, in which paired feeding involves marked restriction of food intake.

Since reductions in hepatic catalase were obtained by treatment with growth hormone, it appeared of interest to determine whether or not any changes in hemoglobin content of the blood occurred. Though not functionally related to catalase, hemoglobin is chemically related in that it contains the porphyrin ring as a prosthetic group. Cell volume determinations

TABLE 4. THE EFFECT OF GROWTH HORMONE ON THE HEMOGLOBIN AND HEMATOCRIT VALUES OF THE BLOOD OF HYPOPHYSECTOMIZED AND NORMAL RATS

Experiment	Hematocrit (vol. %)	Hemoglobin (gm./100 ml.)
No. I in Table 2		
A. Hypophysectomized		
1. Treated	—	11.7 ± 0.6
2. Controls	43. ± 2.5	13.6 ± 0.7
B. Normal		
1. Treated	48. ± 2.9	14.2 ± 0.9
2. Controls	50. ± 1.7	15.6 ± 0.3
No. II in Table 2		
A. Hypophysectomized		
1. Treated	38.6 ± 2.7	12.8 ± 0.8
2. Controls	39.0 ± 1.6	13.1 ± 0.5
B. Normal		
1. Treated	45.5 ± 2.2	15.3 ± 0.6
2. Controls	46.6 ± 1.2	15.6 ± 0.5

were also carried out on the same blood samples. The hypophysectomized controls (Table 4) show the low grade anemia associated with this operation. Treatment of these animals with growth hormone resulted in a further decrease in the hemoglobin values. Certainly, the values show no tendency to return to normal; rather, so far as hemoglobin concentration is concerned, the anemia tends to become worse. As indicated in the next section, the fall in hemoglobin concentration in both normal and operated groups after treatment may be misleading.

DISCUSSION

If one considers enzymes as proteins which can be determined individually, the present and previously published studies of this series clearly indicate that growth hormone does not cause a universal increase in protein synthesis. Hypophysectomy of white rats resulted in lowered tibial alkaline phosphatase, hepatic D-amino acid oxidase and renal alkaline phosphatase; renal and hepatic acid phosphatase, renal D-amino acid oxidase and muscle succinic acid dehydrogenase were unchanged, while hepatic alkaline phosphatase and hepatic catalase increased. Treatment of the operated animals with large doses of growth hormone reversed all changes except that of hepatic D-amino acid oxidase, which remained low. Where no change occurred after hypophysectomy, treatment with growth hormone also produced none. In normal animals, the only enzyme affected by such treatment was hepatic catalase. During growth in the morphological sense, some structures develop while others undergo involution, and the same appears to be true of growth in terms of protein formation. The term "somatotrophin" is perhaps a fortunate one in that it implies an over-all increase in size without suggesting universal acceleration of synthetic activity.

A reciprocal relationship between the 2 heme-containing proteins, catalase and hemoglobin, appears possible. After hypophysectomy, an anemia develops and hepatic catalase increases greatly. That hemoglobin concentration fell further when hepatic catalase was reduced by treatment with large doses of growth hormone may be deceptive. As shown in Table 4, treatment caused the hemoglobin concentration to fall from 13.6 to 11.7, and from 13.1 to 12.8, or 14% and 2.3% respectively, in the 2 groups of hypophysectomized rats. The gains in weight resulting from treatment of these animals (Table 2) were 34.4% and 26.1% respectively. The total hemoglobin of the animals could, therefore, have increased. We hope to do additional studies in which the possibility of a reciprocal relationship between these heme-containing proteins is tested further.

The anemia of hypophysectomy is a well-established fact (Meyer *et al.*, 1937; Gordon and Charipper, 1947; Daughaday *et al.*, 1948; Crafts, 1949). There are good indications that deficiencies of thyrotrophic, adrenocorti-

cotrophic, and gonadotrophic hormones are mainly involved. Our results indicate that any hemopoietic effect which growth hormone may have, when given alone, is not sufficient to restore or even maintain the hemoglobin concentration during the ensuing gain in weight.

It is clear that physiological amounts of pituitary hormones maintain hepatic catalase at its normal level in untreated intact animals. To what extent the growth hormone is involved is not yet certain. Daily doses of growth hormone which restored or maintained normal hepatic catalase values (Table 2) were about 20 times as large, and were continued about twice as long, as those which produced considerable gain in weight without greatly affecting the enzyme (Table 3). Since adrenalectomy appears to diminish hepatic catalase in rats (Begg and Reynolds, 1950), lowering of this enzyme by large doses of growth hormone cannot be ascribed to possible contamination of the preparation with traces of adrenocorticotrophic hormone. It is conceivable that one or more pituitary hormones exert an indirect effect, by altering the amount of a tissue component which reduces hepatic catalase. A substance of this type has been extracted from tumor tissue (Greenfield and Meister, 1950).

SUMMARY

Under stated experimental conditions, concentrations of hepatic catalase in hypophysectomized immature female Sprague-Dawley rats were found to be 2 to 3 times as great as in normal pair-fed controls. This difference was not due to diminution of the enzyme in the normal groups as a consequence of restricted food intake, for it was also observed when hypophysectomized animals were compared with normals fed *ad lib.* Injection of a fairly pure growth hormone preparation in doses of 20 gammas per animal per day for 10 days did not markedly lower the high hepatic catalase of 5 operated animals. In other operated groups hepatic catalase values were reduced to or maintained at normal levels by treatment for 17 to 24 days with a less purified growth hormone preparation, in doses 20 times greater in potency and 50 times greater in weight than those of the purer preparation. Similar large doses produced more variable, though at times significant, small decreases of hepatic catalase in normal young and adult animals.

Treatment with growth hormone had a slight tendency to reduce the hemoglobin concentration of the blood of both normal and hypophysectomized rats. It would appear that lack of growth hormone is not an important causative factor in the anemia of hypophysectomy, the occurrence of which is again confirmed. The rise in hepatic catalase during development of this anemia suggests the possibility of a reciprocal relationship between heme-containing proteins.

Growth hormone preparations do not accelerate protein synthesis in gen-

eral, since the concentrations of some enzymes are increased, while others are unaffected or decreased.

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QUANTITATIVE EFFECTS OF THYROTROPHIC HORMONE ON I^{131} ACCUMULATION IN THYROID AND PLASMA PROTEINS OF HYPOPHYSECTOMIZED RATS*

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THE tyrotrophic hormone (TSH) of the adenohypophysis influences the rate of accumulation of injected I^{131} in the thyroid and in the plasma proteins. After hypophysectomy in the rat, the capacity of the thyroid to trap I^{131} is decreased (Morton *et al.*, 1942; Leblond and Süe, 1941) and the concentration of I^{131} in the thyroxine fraction of the plasma is reduced (Morton *et al.*, 1942). In contrast, administration of TSH increases the ability of the thyroid of man (Reiss *et al.*, 1946; Stanley and Astwood, 1949), the chicken (Larson *et al.*, 1945), the rabbit (Hertz *et al.*, 1940), the rat (Leblond and Süe, 1940) and the guinea pig (Hamilton and Soley, 1940; Leblond and Süe, 1940; Morton *et al.*, 1941) to trap I^{131}, and increases the rate of accumulation of organically bound I^{131} in the plasma of the rat and the guinea pig (Chaikoff *et al.*, 1947). These aforementioned studies have been confined to the action of TSH in the intact organism. The present studies have been conducted in *hypophysectomized* animals and are concerned with the quantitative and temporal aspects of the effect of TSH on the I^{131} trapping-capacity of the thyroid and with the effect of the hormone on the rate of accumulation of protein-bound I^{131} (PBI^{131}) in plasma.

METHODS AND MATERIALS

Male rats from the Sprague-Dawley farm, weighing between 120 and 200 g., were employed. Hypophysectomy was performed by the parapharyngeal technic. The animals had food and water available at all times. The food was a mixture of calf-builder meal (94 per cent), wheat germ (3 per cent) and dried brewers' yeast (3 per cent).

Carrier-free I^{131} was diluted with 0.9 per cent sodium chloride solution and injected intraperitoneally in a dose of 20 microcuries per rat, contained in a volume of 0.5 ml.

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A purified preparation of TSH[1] and a crude extract of hog pituitary tissue were employed in these studies. The purified preparation of TSH was described as having a potency of 13 Junkmann-Schoeller units per mg. It was dissolved in 0.9 per cent sodium chloride solution and stored in the frozen state during the 3-day injection period. Aliquots were thawed just prior to each injection. The volume injected was 0.5 ml. The crude extract was prepared by suspending ground lyophylized hog pituitary tissue[2] in 0.9 per cent sodium chloride solution; the suspension was prepared immediately before each injection. The volume injected was 0.5 ml.

At the time of sacrifice, 4 to 5 ml. of blood were withdrawn into a heparinized syringe, from the vena cava of the rat under sodium pentobarbital anesthesia. A 0.2 ml. aliquot of the plasma was mixed with 0.2 ml. of a 1.0 per cent sodium iodide solution made alkaline to 2N with sodium hydroxide for the determination of total plasma I^{131}. The mixture was evaporated in a cupped planchet and the radioactivity measured in a Geiger-Müller counter.

PBI^{131} was determined as follows. A 1.0 ml. aliquot of plasma was mixed with 9.0 ml. of 10 per cent trichloroacetic acid and centrifuged. The precipitate was washed with 5 ml. of 10 per cent trichloroacetic acid and centrifuged. The washed precipitate was then dissolved in a 1.0 per cent sodium iodide solution made alkaline to 2N with sodium hydroxide, and transferred with rinsings to a cupped planchet. The material was dried and the radioactivity determined in the usual manner.

Following withdrawal of blood the thyroid with underlying trachea was removed and suspended in 10 ml. of 1.0 per cent solution of sodium iodide made alkaline to 2N with sodium hydroxide. The thyroid tissue was completely dissolved after standing in this solution overnight at room temperature. A 0.1 ml. aliquot of the solution was evaporated in a cupped planchet and the radioactivity measured.

TABLE 1. EFFECT OF TIME ELAPSED AFTER HYPOPHYSECTOMY ON UPTAKE OF I^{131} BY THE THYROID. THYROIDS WERE REMOVED 4 HOURS AFTER INTRAPERITONEAL INJECTION OF 20 MICROCURIES OF I^{131}

No. rats	No. days after hypophysectomy	Per cent administered I^{131} trapped by thyroid Average ± std. error
6	Intact	11.31 ± 0.89
5	1	6.50 ± 0.1[illegible]
4	3	5.66 ± 0.75
6	5	4.04 ± 0.79
1	7	3.15
6	11	3.33 ± 0.79

RESULTS

I. *Uptake of I^{131} by the thyroid.*

A. *Effect of hypophysectomy on the uptake of I^{131} by the thyroid: temporal relations.* In the four hours which followed the administration of 29 microcuries of I^{131}, the thyroid of the intact rat trapped 11 per cent of the radio isotope (Table 1). Twenty-four hours after hypophysectomy the capacity of

[1] Thyrotrophic hormone preparation Rx099802 was obtained through the generosity of Mr. L. W. Donaldson and Dr. D. A. McGinty of Parke, Davis & Co.

[2] Gift of Dr. E. E. Hays of the Armour Laboratories.

the gland to trap I^{131} had been reduced to almost 50 per cent of normal; with time, there was a further but very gradual decrease to approximately 30 per cent of normal in the capacity of the gland to trap I^{151}. A low value was reached by the fifth day and hence in subsequent experiments, for convenience, 5 days were allowed to elapse between hypophysectomy and sacrifice of the animal.

B. *Effect of various single doses of TSH on the thyroid uptake of I^{131}.* The results of this study are given in Table 2. TSH was always injected 12 hours prior to I^{131} administration. A single dose of 0.01 mg. had no effect upon the avidity of the thyroid for the radioisotope. Doses of 0.10, 1.0 and

TABLE 2. EFFECT OF TSH ON UPTAKE OF I^{131} BY THYROID OF HYPOPHYSECTOMIZED RATS 5 DAYS AFTER HYPOPHYSECTOMY. THYROIDS WERE REMOVED 4 HOURS AFTER INTRAPERITONEAL INJECTION OF 20 MICROCURIES OF I^{131}. TSH WAS ADMINISTERED SUBCUTANEOUSLY 12 HOURS BEFORE I^{131}

No. rats	Dose TSH, mg.	Per cent administered I^{131} trapped by thyroid Average ± std. error
4	0	4.04 ± 0.79
5	0.01	4.00 ± 0.12
5	0.10	7.26 ± 1.34
5	1.00	13.02 ± 1.74
4	10.00	14.28 ± 0.67

10.0 mg. of TSH induced a significant increase in uptake of I^{131} by the thyroid. The data suggest that 1.0 mg. of TSH is a maximally effective dose. The relatively small increase in I^{131} uptake which followed a single large dose of TSH prompted a study of the effect of divided doses given over different periods.

C. *Exploratory studies designed to test the influence of various dose schedules of TSH on the uptake of I^{131} by the thyroid.* In a trial experiment a total dose of 1.0 mg. of TSH divided into 4 equal portions and injected 12 hours apart, was employed. The first dose was injected 72 hours after hypophysectomy and the last was injected 12 hours before I^{131} administration. The thyroids of the treated rats collected 31.10 ± 3.28 per cent of the injected 20 microcuries of I^{131}, whereas the thyroids of the uninjected controls collected 5.80 ± 1.26 per cent. The value of 31.10 per cent is to be compared with that of 13.02 per cent for the rats injected with a single dose of 1.0 mg of TSH.

It was decided to sacrifice the animals 24 hours after I^{131} administration instead of 4 hours as in the previously described experiments. Examination of Table 3 shows that the accumulation of I^{131} in the thyroid of the untreated hypophysectomized rat is not significantly greater after 24 than after 4 hours. However, in TSH-treated rats there is a greater accumulation in 24 than in 4 hours when comparison is made between rats on similar dose schedules.

Table 3 presents the results of exploratory studies in which the animals were sacrificed 24 hours after I^{131} administration and in which either the last or the penultimate dose of TSH was injected just prior to the administration of I^{131}. From the results of these studies it was decided to employ the 8-hour injection schedule, with certain minor modifications, in subsequent experiments designed to uncover the relation between dose of TSH and capacity of the thyroid to trap I^{131}.

TABLE 3. EFFECT OF TSH ON THE UPTAKE OF I^{131} BY THE THYROID OF HYPOPHYSECTOMIZED RATS. TSH WAS ADMINISTERED SUBCUTANEOUSLY IN EQUALLY DIVIDED DOSES AS INDICATED, 24, 12 OR 8 HOURS APART. THYROIDS WERE REMOVED 24 HOURS AFTER INTRAPERITONEAL INJECTION OF 20 MICROCURIES OF I^{131}

No. rats	Condition of experiment	Per cent administered I^{131} trapped by thyroid Average ± std. error
9	Hypophysectomized control	5.47 ± 0.47
6	One mg. TSH administered in 5 equally divided doses, 24 hours apart. The first dose was injected 24 hours after hypophysectomy and the last dose was injected just prior to I^{131}.	36.42 ± 3.79
8	One mg. TSH administered in 5 equally divided doses, 12 hours apart. The first dose was injected 72 hours after hypophysectomy and the last dose was injected just prior to I^{131}.	44.34 ± 2.45
5	One mg. TSH administered in 8 equally divided doses, 8 hours apart. The first dose was injected 72 hours after hypophysectomy, the 7th dose just prior to I^{131}, and the 8th and last dose 8 hours later.	58.18 ± 6.06

D. *Effect of various doses of TSH, injected in divided portions, on the uptake of I^{131} by the thyroid.* The results of this study are presented in Table 4. A dose of TSH as small as 0.01 mg. produced an appreciable increase in the ability of the thyroid of the hypophysectomized rat to trap I^{131}. A dose of 0.10 mg. produced only a slightly greater response than 0.01 mg. However, between the 0.10 and 0.50 mg. doses there was a sharp rise in the percentage uptake of I^{131} by the thyroid. A maximum response was obtained at the 0.50 mg. dose level. This dose level restores to normal the capacity of the thyroid of the hypophysectomized rat to take up I^{131}. [Compare uptake figures with those of intact untreated controls in Table 4. Also note that the uptake of I^{131} by the thyroid of the intact animal varied considerably (23.23 to 61.53 per cent).] These data are illustrated in Figure 1.

The statistics, s (standard deviation), b (slope of the log dose-response curve) and λ (s divided by b, a measure of the accuracy of the assay method), for the data in Table 4 have been calculated for that part of the dose-response curve which lies between the 0.10 and 0.50 mg dose levels. It is this region of the curve which would be suitable for bioassay purposes.

TABLE 4. EFFECT OF TSH ON THE THYROID UPTAKE OF I^{131} AND ON THE PLASMA CONCENTRATION OF TOTAL I^{131} AND PBI^{131} IN HYPOPHYSECTOMIZED RATS. TSH WAS ADMINISTERED SUBCUTANEOUSLY IN THE INDICATED TOTAL DOSE IN 8 EQUALLY DIVIDED PORTIONS, 8 HOURS APART. THE FIRST DOSE WAS INJECTED 72 HOURS AFTER HYPOPHYSECTOMY, THE 7TH DOSE JUST PRIOR TO I^{131} AND THE 8TH DOSE 8 HOURS LATER. THYROIDS WERE REMOVED AND BLOOD WAS WITHDRAWN 24 HOURS AFTER INTRAPERITONEAL INJECTION OF 20 MICROCURIES OF I^{131}

Dose TSH, mg.	Per cent administered I^{131} trapped by thyroid Average ± std. error	Per cent administered I^{131} in 1 ml. plasma	
		Total I^{131} Average ± std. error	PBI^{131} Average ± std. error
0 (intact)	44.64 ± 5.03 (8)*	.0672 ± .0103 (8)*	.02350 ± .00667 (8)*
0 (hypox)	7.86 ± 0.75 (14)	.1845 ± .0103 (14)	.00396 ± .00025 (14)
0.01	16.17 ± 1.04 (5)	.1698 ± .0031 (5)	.00146 ± .00033 (5)
0.10	20.79 ± 1.42 (5)	.1641 ± .0002 (6)	.00214 ± .00019 (4)
0.25	33.81 ± 1.92 (11)	.1121 ± .0064 (11)	.00610 ± .00246 (11)
0.50	56.26 ± 2.81 (6)	.0978 ± .0002 (7)	.02040 ± .00178 (7)
0.75	54.61 ± 3.89 (7)	.0782 ± .0019 (7)	.03966 ± .00835 (3)
1.00	53.18 ± 3.80 (14)	.0746 ± .0022 (14)	.03980 ± .00062 (10)
10.00	51.30 ± 1.13 (8)	.2572 ± .0039 (8)	.04286 ± .00038 (7)

For the thyroid I^{131} uptake data, the following statistics apply to the responses to the 0.10, 0.25 and 0.50 mg. dose levels: $b = 47.1$; $s = 7.00$; $\lambda = 0.149$.

For the PBI^{131} data, the following statistics apply to the responses to the 0.25, 0.5 and 1 mg. dose levels: $b = 0.0558$; $s = 0.0118$; $\lambda = 0.221$.

* Figures in parentheses indicate the number of animals.

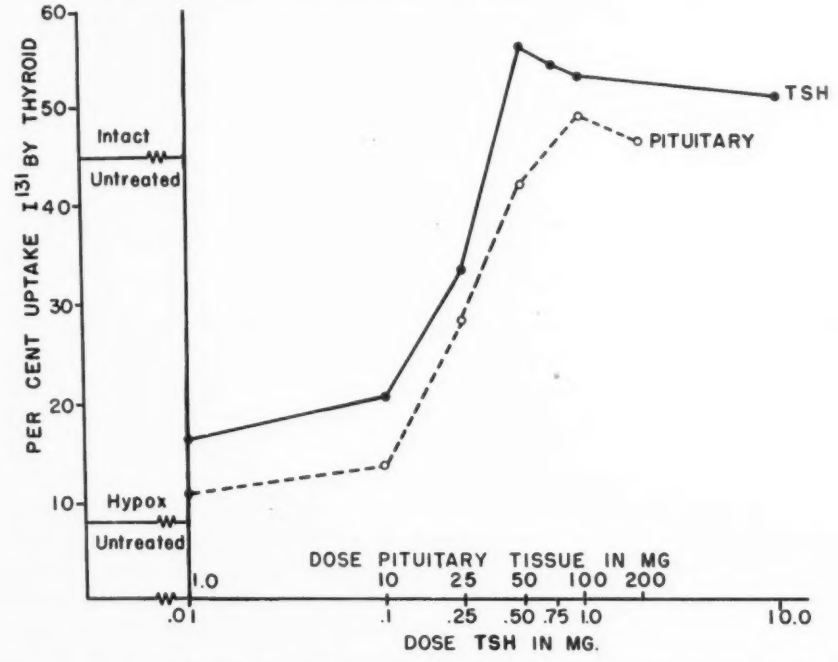

FIG. 1. Effect of purified TSH and crude extract of hog pituitary on uptake of I^{131} by thyroid of hypophysectomized rats. To the left of the figure are presented values for intact and hypophysectomized untreated animals.

The λ value indicates that uptake of I^{131} by the thyroid of the hypophysectomized rat is an accurate method for the bioassay of TSH.

E. *Effect of crude extracts of hog pituitary tissue on the uptake of I^{131} by the thyroid.* A crude extract of hog pituitary tissue was injected according to the schedule employed for purified TSH in Table 4. The results are presented in Table 5. It appears that the shape of the log dose-response curve is the same for the crude extract as for purified TSH. (There is no significant difference between the slopes of the two log dose-response curves, $P = 0.2$.) This suggests that it is the TSH fraction of the crude extract which is responsible for the changes in uptake of I^{131} by the thyroid. The lyophilized

TABLE 5. EFFECT OF HOG PITUITARY EXTRACT ON THE THYROID UPTAKE OF I^{131} AND ON THE PLASMA CONCENTRATION OF TOTAL I^{131} AND PBI^{131} IN HYPOPHYSECTOMIZED RATS. PITUITARY EXTRACT WAS ADMINISTERED SUBCUTANEOUSLY IN THE INDICATED TOTAL DOSE, IN 8 EQUALLY DIVIDED PORTIONS, 8 HOURS APART. THE FIRST DOSE WAS INJECTED 72 HOURS AFTER HYPOPHYSECTOMY, THE 7TH DOSE JUST PRIOR TO I^{131} AND THE 8TH DOSE 8 HOURS LATER. THYROIDS WERE REMOVED AND BLOOD WAS WITHDRAWN 24 HOURS AFTER INTRAPERITONEAL INJECTION OF 20 MICROCURIES OF I^{131}

Dose pituitary extract mg.	Per cent administered I^{131} trapped by thyroid Average ± std. error	Per cent administered I^{131} in 1 ml. plasma	
		Total I^{131} Average ± std. error	PBI^{131} Average ± std. error
0	7.86 ± 0.75 (14)*	.1845 ± .0103 (14)*	.00396 ± .00025 (14)*
1	10.92 ± 1.01 (5)	.0625 ± .0022 (5)	.00138 ± .00018 (5)
10	13.27 ± 0.74 (5)	.0694 ± .0151 (5)	.00292 ± .00010 (4)
25	28.61 ± 3.21 (10)	.0506 ± .0071 (6)	.00373 ± .00094 (6)
50	42.13 ± 6.48 (4)	.0643 ± .0216 (5)	.00430 ± .00020 (4)
100	49.34 ± 4.18 (5)	.0776 ± .0144 (5)	.01848 ± .00725 (5)
200	46.69 ± 2.29 (4)	.1170 ± .0149 (5)	.03557 ± .00627 (4)

For the thyroid I^{131} uptake data, the following statistics apply to the responses to the 10, 25, 50, and 100 mg. dose levels: $b = 36.9$; $s = 9.41$; $\lambda = 0.255$.

For the PBI^{131} data, the following statistics apply to the responses to the 50, 100 and 200 mg. dose levels: $b = 0.0522$; $s = 0.0118$; $\lambda = 0.224$.

* Figures in parentheses indicate the number of animals.

hog pituitary has approximately one per cent of the potency of purified TSH. These data are illustrated in Figure 1. The statistics s, b, and λ, for the crude extract are presented in Table 5. They are quite similar to those for purified TSH (Table 4).

II. *Plasma I^{131}*

A. *Effect of purified TSH.* Total I^{131} and PBI^{131} were determined on plasma samples from the rats for which thyroid I^{131} data have already been presented. The results of the analyses are presented in Table 4. No increase in the concentration of PBI^{131} occurred at the 0.01 and 0.10 mg. dose levels; a dose of 0.25 mg. produced an increase which was not statistically significant. Between the 0.25 and the 0.75 mg. dose levels there was a precipitous increase in the concentration of PBI^{131}. An unequivocal maximum response occurred at the 0.75 mg. dose level. A dose of 0.5 mg.

restored the concentration of PBI^{131} to a value equal to that of untreated intact controls (Table 4). These data are illustrated in Figure 2.

The statistics for the data on PBI^{131} are presented in Table 4. The λ value, 0.211, indicates that the effect of TSH on PBI^{131} in the hypophysectomized rat is of potential value for the accurate assay of the hormone.

The total I^{131} of the plasma decreased as the PBI^{131} increased, except that after the 10.0 mg. dose of purified TSH the concentration of total I^{131} in plasma was actually greater than in the untreated hypophysectomized

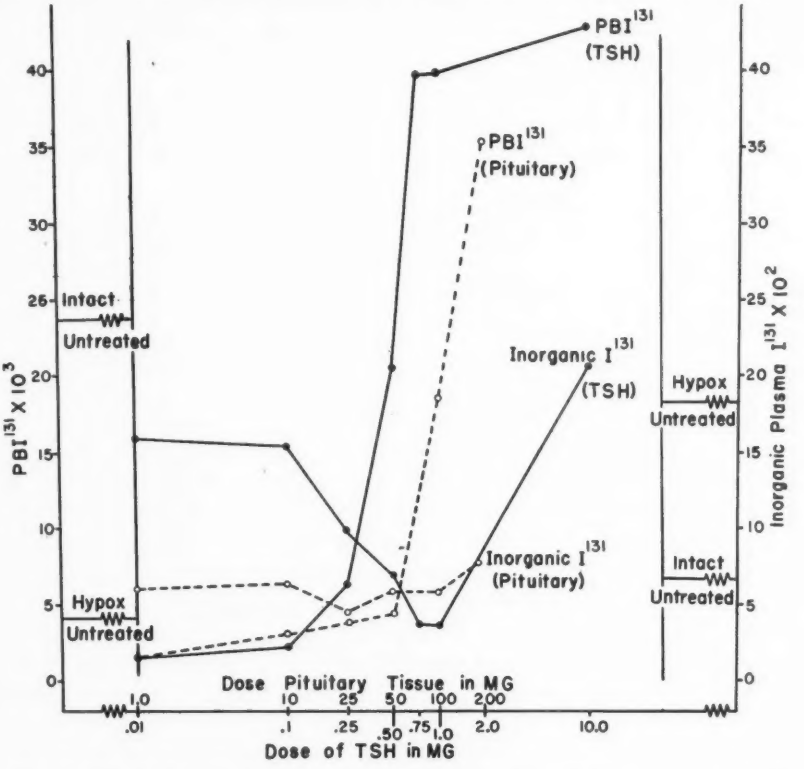

FIG. 2. Effect of purified TSH and crude extract of hog pituitary on concentrations of PBI^{131} and inorganic plasma I^{131} in hypophysectomized rats. To the left of the figure are presented concentrations of PBI^{131} and to the right, concentrations of inorganic plasma I^{131} in intact and hypophysectomized untreated rats. The ordinate for PBI^{131} is plotted as the per cent of the injected dose of I^{131} which accumulated in the proteins of 1.0 ml. of plasma times one thousand. The ordinate for inorganic plasma I^{131} is plotted as the per cent of the injected dose of I^{131} which accumulated in 1.0 ml. of plasma times one hundred. Inorganic plasma I^{131} is the difference between total I^{131} and PBI^{131} (tables 4 and 5).

controls. The abnormally high concentration of total plasma I^{131} in untreated hypophysectomized rats compared to intact rats is associated with the poor capacity of the thyroid to trap I^{131}. Doses of 0.5 to 0.75 mg of TSH restored the concentrations of total plasma I^{131} and PBI^{131} to values approximating those found in untreated intact rats (Table 4). (See Figure 2.)

B. *Effect of crude extract of hog pituitary tissue.* A crude extract of hog pituitary increased the plasma concentration of PBI^{131} in the rats for which analyses of thyroid uptake of I^{131} have already been presented. The results of the PBI^{131} study are presented in Table 5. The crude extract did not induce a significant increase in PBI^{131} concentration in doses less than 100 mg. at which dose level there was more than a four-fold increase in the concentration of PBI^{131}. A dose of 200 mg. produced an even greater effect. Still larger amounts should be tested in order to determine the dose which will produce a maximum response. These results are illustrated in Figure 2.

The statistics, s, b and λ, presented in Table 5 are similar to those for purified TSH in Table 4. There is no significant difference between the slopes of the log dose-response curves for the purified and the crude preparation. This suggests that it is the TSH fraction of the crude extract which is responsible for the increase in concentration of PBI^{131}.

A dose of 1.0 mg. of crude pituitary extract produced a relatively marked reduction in total plasma I^{131} when compared with 0.01 mg. of purified TSH. This is unexpected since the 1.0 mg. dose of crude extract was actually less effective than 0.01 mg. of TSH in inducing an increase in the uptake of I^{131} by the thyroid. It is obvious that the ability of crude extract to induce an increased uptake of I^{131} by the thyroid does not parallel its ability to reduce plasma inorganic I^{131}. A factor other than TSH appears to be present in pituitary tissue which influences total plasma I^{131}. Doses of crude pituitary extract greater than 1.0 mg. produced no further significant decrease in total plasma I^{131}. The slight increases in total plasma I^{131} which occurred at the 100 and 200 mg. dose levels are accounted for in large measure by the changes which these doses produced in the concentration of PBI^{131}.

III. *Changes in the thyroid*

A. *Thyroid weight.* The effect of purified TSH on thyroid weight, over the same dose range employed in the thyroid I^{131} and the PBI^{131} experiments, was studied in a separate group of rats. The results are presented in Table 6. A dose of 0.5 mg. of TSH produced, a significant increase in thyroid weight. A dose of 10.0 mg. almost doubled thyroid weight. Larger amounts must be tried in order to determine the dose which will produce a maximum response.

The statistics, s, b and λ, suggest that the thyroid weight response is not as accurate a method of assay as the uptake of I^{131} by thyroid or the accumulation of I^{131} in plasma protein.

B. *Height of thyroid cells.* The results of this study are presented in Table 6. The data presented here should not be used as an index of the accuracy of this method since the number of cells measured per thyroid was only 6, a number admittedly too small to make an accurate estimate of average cell height. The purpose of measuring thyroid weight and cell height was not to assess these responses with regard to possible usefulness for assay but to correlate the changes in these indices of thyroid function with uptake of I^{131} by the thyroid and with concentration of PBI^{131}. The

TABLE 6. EFFECT OF TSH ON THYROID WEIGHT AND CELL HEIGHT IN HYPOPHYSECTOMIZED RATS. TSH WAS ADMINISTERED SUBCUTANEOUSLY IN THE INDICATED TOTAL DOSE IN 8 EQUALLY DIVIDED PORTIONS, 8 HOURS APART. THE FIRST DOSE WAS INJECTED 72 HOURS AFTER HYPOPHYSECTOMY. THYROIDS WERE REMOVED 16 HOURS AFTER THE ADMINISTRATION OF THE LAST DOSE OF TSH

Dose TSH, mg.	Thyroid weight Average ± std. error mg.	Thyroid cell height Average ± std. error microns
0	7.30 ± 0.42 (7)*	5.03 ± 0.36 (9)*
0.01	7.26 ± 0.23 (5)	4.43 ± 0.26 (6)
0.10	7.66 ± 0.57 (5)	5.67 ± 0.28 (7)
0.25	8.59 ± 0.70 (5)	6.47 ± 0.35 (6)
0.50	9.55 ± 0.43 (6)	9.02 ± 0.57 (6)
0.75	12.51 ± 0.62 (7)	9.68 ± 0.61 (6)
1.00	11.83 ± 0.50 (7)	10.68 ± 0.68 (8)
10.00	13.87 ± 1.11 (6)	14.70 ± 0.33 (6)

The following statistics apply to the data on the response of the thyroid weight to the 0.10, 0.25, 0.50, 0.75, 1.0 and 10.00 mg. dose levels; $b = 2.74$; $s = 1.12$; $\lambda = 0.410$.

* Figures in parentheses indicate the number of animals.

changes in cell height closely paralleled the changes in thyroid weight. However, whereas a dose of 0.25 mg. produced a significant increase in cell height, gland weight did not show a significant increase until a dose of 0.5 mg. was employed. As in the case of weight, cell height showed a gradual increase with increasing dose, in contrast with the precipitous alterations in uptake of I^{131} by the thyroid and in concentration of PBI^{131}.

DISCUSSION

A single intraperitoneal dose of 30 microcuries of I^{131} has been shown by Feller *et al.* (1949) to have no demonstrable deleterious effect on the thyroid function of the rat, as judged by a number of criteria. It is relatively certain that in the present study, the radiation from that fraction of the injected 20 microcuries of I^{131} which accumulated in the thyroid had no influence upon the function of the gland and that the alterations observed were caused by hormone treatment.

The results of the present study confirm previous findings that hypophysectomy markedly reduces but does not completely prevent the thyroid from trapping I^{131} (Morton *et al.*, 1942). They are in conformity with the findings of Vanderlaan and Greer (1950) that the iodide-concentrating mechanism of the thyroid is dependent on the functional activity of the pituitary. The higher than normal concentration of inorganic I^{131} in the plasma of the hypophysectomized rat reflects the impairment in the capacity of the thyroid to trap I^{131}. In addition, the results confirm the observation that removal of the pituitary decreases the accumulation of I^{131} in the organically bound iodine fraction in the plasma (Morton *et al.*, 1942).

The experiments herein reported represent the first study of the temporal and quantitative aspects of the effect of TSH on the uptake of I^{131} by the thyroid and on the concentration of I^{131} in the plasma proteins in the hypophysectomized animal. Statistical analyses indicate that both phenomena are potentially useful bioassay technics. Final assessment of their value in this regard must await their application to practical assay problems. It would be premature to compare these assay technics with presently employed procedures. However, preliminary studies in this laboratory indicate that uptake of I^{131} by the thyroid and concentration of PBI^{131} represent satisfactory assay methods from the standpoint of reproducibility and accuracy. They are relatively simple and are not unduly laborious. However, they are definitely less sensitive than several presently employed methods.

The procedures appear to fulfill the following criteria for an acceptable assay of TSH as outlined by Albert (1949), namely, specificity, objectivity and precision. They do not fulfill his requirements of convenience and sensitivity. According to Albert, "Convenience requires that the assay should be completed within a few hours, or at least during the same day, and not after a week of injections." As stated above, the methods described in this report are definitely not as sensitive as certain currently employed technics (see Albert, 1949). However, the total quantity of material required to gain an estimate of potency is determined by the accuracy as well as the sensitivity of a method. A highly sensitive method, because of low order of accuracy, may require a relatively large total quantity of material to make a reliable estimate of potency; low order of accuracy is usually compensated for by the use of relatively large numbers of test animals.

It is of importance to note that the uptake of I^{131} and the concentration of PBI^{131} in untreated intact rats were almost equal to the values obtained in hypophysectomized rats treated with maximum effective doses of TSH. The thyroid of the intact rat is apparently operating at a nearly maximum level of activity. It is obvious that intact rats are relatively insensitive to

TSH and hence unsuitable test objects for the assay of this hormone.

Increases in thyroid weight and cell height show progressive changes throughout the dose range used, in contrast to the precipitous rise in the dose-response curves for thyroid uptake of I^{131} and for PBI^{131}. Steep rectilinear log dose-response curves are an advantage for accuracy of assay.

The decrease in plasma inorganic I^{131} during the period of TSH-induced increases in thyroid uptake of I^{131} is to be expected and is in harmony with the fact that after a tracer dose of radioiodine the activity of the inorganic fraction (Potts *et al.*, 1949) or the total radioiodine (McConahey *et al.*, 1949) of the blood in hyperthyroid patients declines more rapidly than normal, whereas in myxedema the decline is slower than normal. Presumably inorganic iodine is being rapidly incorporated into thyroid hormone under the influence of TSH. The increase of total plasma I^{131} after high doses of TSH or pituitary extract was not anticipated. No explanation is offered at this time.

That PBI^{131} decreases in concentration after the administration of small doses of TSH and pituitary extract is an apparent anomaly. The data of Morton *et al.* (1942) indicate that I^{131} accumulates at a faster rate in the diiodotyrosine fraction of plasma in hypophysectomized than in intact rats; on the other hand, there is a marked decrease below normal in the rate of accumulation of I^{131} in the thyroxine fraction of plasma after hypophysectomy. One may speculate that the reduction in PBI^{131} which follows the administration of small doses of TSH is associated with the correction of an abnormal ratio of thyroxine to diiodotyrosine turnover. Reduction in the concentration of I^{131} in the diiodotyrosine fraction may very well mask an actual increase in the thyroxine I^{131} fraction.

The concentration of I^{131} in the thyroid at any one time is the algebraic sum of rate of accumulation of I^{131} as iodide and rate of discharge of I^{131} as iodide and thyroid hormone. The fact that the concentration of thyroid I^{131} reaches a plateau does not necessarily mean that rate of uptake of I^{131} has reached a maximum. On the contrary, it is quite probable that it indicates that rate of discharge has equaled or overtaken the rate of uptake. Likewise for PBI^{131}, a plateau does not mean that the thyroid has reached a maximum rate of discharge of thyroid hormone. It may very well reflect an increased rate of degradation of the thyroid hormone in the peripheral tissues.

SUMMARY

Hypophysectomy in the rat markedly reduces the capacity of the thyroid to trap I^{131}. TSH restores this capacity to normal. At the same total dose level, single injections of TSH are less effective than multiple injections in increasing the uptake of I^{131} by the thyroid of the hypophysectomized rat. The form and statistics of the log dose-response curve indicate

that uptake of I^{131} by the thyroid in hypophysectomized rats, treated with multiple injections of TSH, is a potentially useful method of assay for the hormone. The slopes of the log dose-response curves for purified TSH and for a crude extract of hog pituitary are not significantly different.

Hypophysectomy reduces the rate of accumulation of I^{131} in the plasma proteins of the rat. Multiple injections of TSH restore the capacity of the plasma proteins of the rat to accumulate I^{131}. The form and statistics of the log dose-response curve indicate that the TSH-induced increase in concentration of PBI^{131} in hypophysectomized rats is a potentially useful method of assay for the hormone. The slopes of the log dose-response curves for purified TSH and for a crude extract of hog pituitary tissue are not significantly different.

Following the administration of small and moderately large doses of purified TSH the concentration of plasma inorganic I^{131} decreases as the uptake of I^{131} by the thyroid increases. Very large doses of TSH increase plasma inorganic I^{131} to abnormally high levels.

Crude pituitary extract has a relatively marked effect on decreasing plasma inorganic I^{131} as compared to its action on uptake of I^{131} by the thyroid.

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